UNIVERSITY OF LIVERPOOL

An Investigation into the Combined Tocolytic Effect of Magnesium and Other Tocolytics

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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May 2019

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Acknowledgements

Embarking on an academic journey of this level would certainly be impossible without the invaluable input of many. The last four years has been full of surprises, including having a baby, and so many ups and down. Nevertheless, the support and encouragement of so many have made it possible to complete this journey and I must say I have definitely enjoyed this roller coaster. Therefore, I would like to express my sincere gratitude to my primary supervisor Professor Susan Wray for the excellent role she played during my PhD. She wasn't just a supervisor, but an encourager and at other times a mum. I have been very privileged to have been supervised by her.

I would also like to thank my secondary supervisor Dr Helen Wallace for being a shoulder to lean on. Thanks for always lending me a listening ear even when my moaning didn't always make sense. Special thanks to Dr Sarah Arrowsmith who taught me the skill of dissection and challenged me to think outside the box. I would also like to thank all members of our research group including Dr Clodagh Prendergast, Asmaa Almohanna and Seham Alsaif.

Thanks to my husband and children for putting up with my talk of science and preterm labour. Thanks for those times you had to pretend that you understood what I was talking about. Thanks for your patience with me while I wrote my thesis. Without your patience and support, this wouldn't have been possible. My thanks also go to my amazing sisters who were always on the other side of the phone listening to me moan about physiology. You both rock!

Finally, this PhD is for my Mum and Dad who encouraged me that I could do it no matter the obstacles. Thanks dad for your prayers, and for allowing mum spend four months with me while I wrote my thesis. Mum, for those times you stayed awake all night just to make sure I read, I say thank you. I guess it's safe to say that it was worth it. You are the best!

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List of Abbreviations

ADP	Adenosine-5’-diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine-5’-triphosphate
AUC	Area under the curve
BKCa	Large conductance calcium-activated potassium channel
BP	Base pairs
Ca^{2+}	Calcium ions
Ca^{2+}-CaM	Calcium-calmodulin complex
cAMP	Cyclic adenosine monophosphate
CBS	Cystathionine β-synthase
cGMP	Cyclic guanosine monophosphate
CICR	Calcium induced calcium release
Cl^-	Chloride ions
CSE	Cystathionine γ-lyase
COX	Cyclo-oxygenase
C_t	Threshold cycles
DMSO	Dimethyl sulphoxide
E2	Oestrogen
ECC	Excitation Contraction Coupling
EDTA	Ethylene diamine tetra acetic acid

F+R	Forward and reverse
GPCR	G Protein Coupled Receptor
HEPES	Hydroxyethylpiperazine-N-2-Ethane Sulfonic Acid
IC_{50}	Half maximal inhibitory concentration
ICC	Interstitial cells of Cajal
IK_{Ca}	Intermediate calcium-activated potassium channel
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
K^+	Potassium ions
K_{ATP}	ATP-sensitive potassium channel
KCl	Potassium solution
kDa	Kilo daltons
Kir	Inward-rectifier potassium channels
K_v	Voltage-gated potassium channel
M	Moles
MAPK	Mitogen-activated protein kinase
mg	Milligrams
Mg	Magnesium
MgCl2	Magnesium chloride
$MgSO_4$ $7H_20$	Magnesium sulphate heptahydrate
MIQE	Minimum Information for Publication of quantitative real-time PCR Experiments

MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mM	Millimolar
mN	Millinewton
mRNA	Messenger ribonucleic acid
mV	Millivolts
Na^+	Sodium ions
NaCl	Sodium chloride
NCX	Sodium-calcium exchanger
NICE	National Institute for Health and Care Excellence
nM	Nano molar
NoRT	No reverse transcriptase
NOS	Nitric oxide synthase
NP	Non-pregnant
NTC	No template control
O_2	Oxygen
OTR	Oxytocin receptor
P4	Progesterone (P4)
PG	Prostaglandin
PGF2α	Prostaglandins F2α
pHi	Intracellular pH

pH_o	Extracellular pH
PIP_2	Phosphatidylinositol 2, 5-biphosphate
PLA2	Polylactide
PLC	Phospholipase C
pM	Pico molar
PMCA	Plasma membrane Ca^{2+}-ATPase
PP	Postpartum
PSS	Physiological saline solution
RCOG	Royal College of Obstetricians and Gynaecologists
RT	Reverse transcriptase
RyR	Ryanodine receptors
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
SKCa	Small conductance calcium-activated potassium channels
SOCC	Store operated calcium channels
SOCE	Store operated calcium entry
SR	Sarcoplasmic reticulum
TAE	Tris acetate EDTA
TASK	Two-pore domain acid sensing K^+
TEA	Tetraethyl Ammonium Chloride
TRP	Transient receptor potential
VGCC	Voltage gated calcium channel

$[Ca^{2+}]_i$	Intracellular Concentration of Calcium
µl	Micro litre
µM	Micro molar

Abstract

An investigation into the combined tocolytic effect of magnesium and other tocolytics.

Blessing Osaghae

Preterm births are a leading cause of perinatal morbidity and mortality, and its occurrence has remained unchanged. To improve perinatal mortality, several tocolytics were developed; however, this has not been accompanied by an improvement in infant outcome (Keirse, 2003a). They delay labour long enough to administer corticosteroids.

According to the NICE guidelines (NICE, 2016), women who are at risk of preterm birth before 30 weeks of pregnancy are to be administered magnesium sulphate ($MgSO_4$) to help reduce the risk of cerebral palsy. So, although $MgSO_4$ is not currently used for the treatment of preterm labour in the UK, it is being administered clinically in combination with a tocolytic which could either be indomethacin, nifedipine, or atosiban. This means that in certain preterm cases (<30 weeks), a dual tocolytic therapy would be given

The aims of this thesis were mainly to: (1) investigate the effect of magnesium sulphate at different gestations of pregnancy and how hormonal changes may affect its efficacy; (2) to investigate the dual tocolytic effect of magnesium sulphate ($MgSO_4$) and other tocolytics (atosiban, indomethacin and nifedipine) (3) to determine the expression of L-type calcium channels especially the α1c subunit (Cav 1.2) across different gestational states of mouse, and in doing so, (4) establish a panel of stable housekeeping genes that could be used across gestational states of mouse myometrium.

Magnesium sulphate concentration-dependently decreased spontaneous and oxytocin-induced contractions. In spontaneous contractions, $MgSO_4$ showed a more potent effect at term and the least effect in non-pregnant tissues. In the presence of oxytocin however, there was no difference in the potency of $MgSO_4$ across all gestations studied. Having established the effect of $MgSO_4$ on my samples, I then investigated its combined effect with indomethacin, atosiban and nifedipine. In the

presence of oxytocin, $MgSO_4$ +atosiban produced a synergistic tocolytic effect on contractions, $MgSO_4$ plus indomethacin produced an antagonistic effect while there was no significant difference in the effect of $MgSO_4$ plus nifedipine compared to $MgSO_4$. Although $MgSO_4$ plus atosiban produced the greatest tocolytic effect compared to other combinations, I found that extracellular acidification reversed its effect, increasing force and frequency of contractions. Since magnesium acts at L-type calcium channels, I investigated and quantified the expression of L-type calcium channel (Cav 1.2) and found that expression increased towards term with further increase postpartum.

From this work I have shown that magnesium concentration-dependently reduces spontaneous contractions but its effect is reduced in the presence of oxytocin which may explain why magnesium is not effective clinically. The synergistic effect seen with $MgSO_4$ plus atosiban suggests that oxytocin receptor antagonists may be the most efficient tocolytics in cases where magnesium is administered. The increase in Cav 1.2 expression towards term indicates its role in parturition.

Chapter 1

General Introduction

Chapter 1

General Introduction

My thesis investigates the effect of magnesium in combination with other tocolytics on *in vitro* uterine contractions. In this introductory chapter, I will describe the anatomy of the uterus (human and mouse). I will then describe the structure of the myometrium, its function and mechanism. The mechanism of contraction would also be covered in this thesis as well as the regulation of intracellular calcium in uterine contractions and the role of the sarcoplasmic reticulum. Furthermore, the process by which hormones modulate force of contraction will be explained as well as the mechanism of action of oxytocin and prostaglandins.

In this thesis, I will describe the prevalence and challenges of preterm birth and labour. I will then review what is known about the tocolytics used in this thesis and their mechanism of action. I will also review the concept of combined tocolytics, the benefit and challenges. In general, this review mainly covers literature on human myometrium and mouse (my experimental model) where possible.

1.1 Uterus

1.1.1 The Human uterus

The human uterus is a hollow, pear-shaped muscular organ and sits inverted within the pelvic region. It is situated between the urinary bladder and the rectum. It measures between 6 to 8 cm long, 5 cm broad and the thickness is about 2 to 3 cm. A typical non-pregnant uterus weighs about 30 - 40 grams but increases greatly in pregnancy (Gray, 2000).

Anatomically, the human uterus comprises mainly of four regions: the fundus, uterine the body, the isthmus and the cervix (Figure 1.1). The fundus is the rounded, broad upper area in which the fallopian tubes are connected to the uterus. The body, otherwise referred to as “the corpus” is the main part of the uterus, which starts below the level of the fallopian tubes and continues downwards until the uterine walls. The cavity begins to narrow; the isthmus is the lower, narrow neck region; and the cervix, which is the lowest section of the uterus, extends from the isthmus until it opens into the vagina.

The walls of the uterus compose of three tissue layers: the perimetrium, the myometrium and the endometrium. The perimetrium is the outer layer, like an external skin of the uterus that protects the uterus from friction. The myometrium forms the middle layer off the uterus, which allows the uterus to expand and contract during pregnancy and labour. The endometrium is the non-excitable layer that borders around the hollow lumen of the uterus.

The uterus is supplied with blood by the two uterine arteries, which are branches of the internal iliac arteries, and by ovarian arteries, which connect with the ends of the uterine arteries and send branches to supply the uterus. The uniqueness of the uterus lies in the sequential changes necessary for completion of pregnancy (Young, 2007).

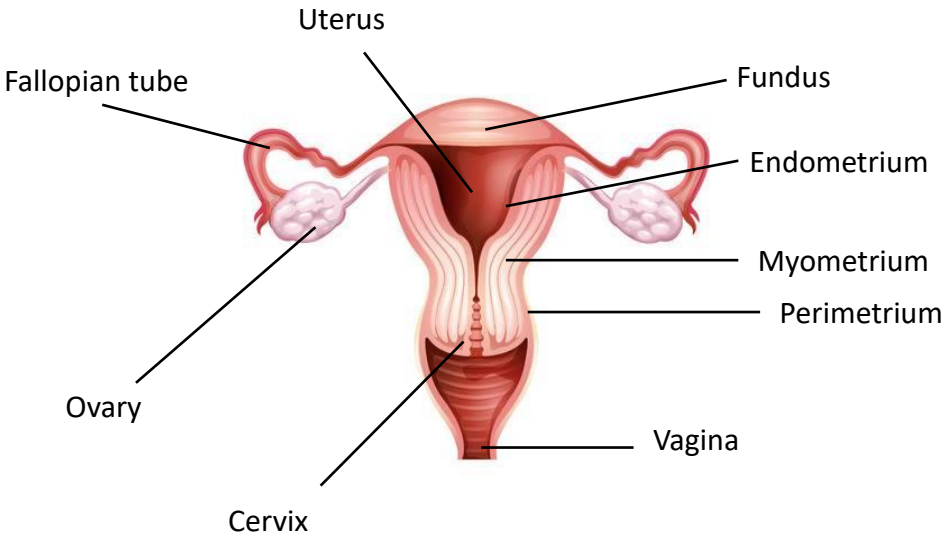


Figure 1. 1: The Anatomy of the Human Uterus

1.1.2 The Mouse uterus

The mouse, like other rodents, has a duplex uterus, consisting of two branches, known as horns enabling multiple offspring to be developed on both horns. The mouse uterus is a duplex unit, consisting of two horns and come together above the cervix. The cervix composes of the caudal region which is narrow and projects into the cranial part of the vagina (Figure 1.2). It contains a single undivided lumen or canal.

Over the years, the mouse has been an excellent model for investigating preterm birth and the general physiology of parturition. Much of our understanding of uterine contraction originates from animal studies, including mouse, rat and baboon (Wray et al., 2001). The study of animal myometrial tissue during pregnancy has its obvious advantages compared with the study of human myometrial tissue which can usually only be obtained during caesarean section or hysterectomy. The vast majority of work on human myometrium come from elective non-labouring women, as it is very difficult to obtain labouring samples due to the context in which the caesarean section arise. Animal myometrium can be acquired at different gestations from pregnant animals, and changes in uterine activity directly measured *in vivo*, so that the switch from quiescence to pre-labour and labour stages can be elucidated (Tribe, 2001).

One advantage in using the mouse is that the short gestation period is convenient for experimentation. Using mouse also allows for generation of knockout models which are beneficial in investigating genes that influence parturition (Ratajczak et al., 2010). The EC coupling and contraction mechanism of mouse myometrium is similar to the human (Matthew et al., 2004a). Both mouse and human myometrial smooth muscles require L-type calcium entry; express many of the same ion channels and transporters that govern excitability and ionic homeostasis (Matthew et al., 2004a). In addition, major components of labour and delivery are maintained in both mice and humans. For example, the expression of certain contraction-associated proteins like prostaglandin, oxytocin, and connexin-43 are all elevated in both human and mice (Ratajczak and Muglia, 2008, Cook et al., 2000).

There are however fundamental differences between mouse and human reproductive anatomy and physiology. While the mouse has a bicornuate uterus, the human uterus has a pear shaped uterus. The mouse also has a larger litter size than humans. In mouse pregnancy, there is a drastic decline in maternal progesterone at the end of pregnancy (Hardy et al., 2006), however this decline is not seen at the onset of human labour (Mendelson, 2009). In humans rather, both progesterone levels and progesterone receptor levels remain elevated throughout pregnancy and into labour (Hardy et al., 2006, Challis et al., 2000). Despite these differences in uterine shape, number of embryos, and site of generation of progesterone in late gestation (Ratajczak et al., 2010), nonetheless, the genetic traceability and basic similarities in EC coupling, makes the mouse an attractive experimental model (Ratajczak and Muglia, 2008) and there remains some similarity in the structural morphology. The uterine wall of the mouse, as with human, consists of the endometrium (the interior layer that lines the body of the uterus), the myometrium (smooth muscle layer) and the perimetrium (the outermost serosa layer).

Unlike humans who menstruate, the uterine lining of the mouse uterus is not shed and passed out of the body. Instead, the endometrial layer is transformed for the next cycle. These periodic changes in the endometrium, as well as changes that occur in the ovaries and vagina, are part of the oestrus cycle of the mouse. In the mouse, the oestrous cycle is divided into 4 stages (proestrus, oestrus, metestrus, and diestrus) and repeats every 4 to 5 days unless interrupted by pregnancy, pseudo-pregnancy, or anoestrus (Byers et al., 2012). The main hormones that regulate the oestrus cycle are: gonadotropin releasing hormone, which is secreted by the hypothalamus and stimulates the release of gonadotropins by the pituitary gland; the gonadotropins luteinising and follicle stimulating hormones secreted by the pituitary gland; oestrogen (E2), progesterone which is synthesised in the ovaries; and prolactin, which is secreted by the pituitary gland following mating (Freeman, 2006). During proestrus, E2-secreting follicles grow under the influence of follicle stimulating hormones, increasing the secretion of E2 (Fortune, 1994; (Fortune, 1994, Maeda et al., 2000). This results in an increase in E2 levels, and

stimulates the release of luteinising hormones. The release of the luteinising hormone in turn results in stimulating ovulation during the oestrus stage. During the oestrus stage, rodents like rats and mice are sexually receptive and mating is possible. Within several hours, ovulation occurs and the follicles are transformed into corpora lutea. During metestrus, the corpora lutea grows post-ovulation, secreting Progesterone (P4). In diestrus stage, the corpora luteal continue to secrete high quantities of P4. It is during this stage of the oestrus cycle that FSH levels begin to increase in the absence of pregnancy, and stimulate the growth of a new set of follicles, which will occur in the proestrus stage of the following cycle (Westwood, 2008, Freeman, 2006).

Blood is supplied to the uterus through the uterine artery which branches out through the myometrium to give off arcuate arteries, then radial arteries and further spiral arteries. The spiral arteries then supply blood to the endometrium, placenta and decidua during pregnancy. In humans, the size of the uterine artery doubles during pregnancy (Palmer et al., 1992) . Similar findings have been shown in animal studies including rodents (K Griendling et al., 1985, Keyes et al., 1997).

As a result of the position and network of the arteries, contraction will cause a compression of the uterine vessels resulting in reduced blood flow. This event leads to hypoxia and pH changes.

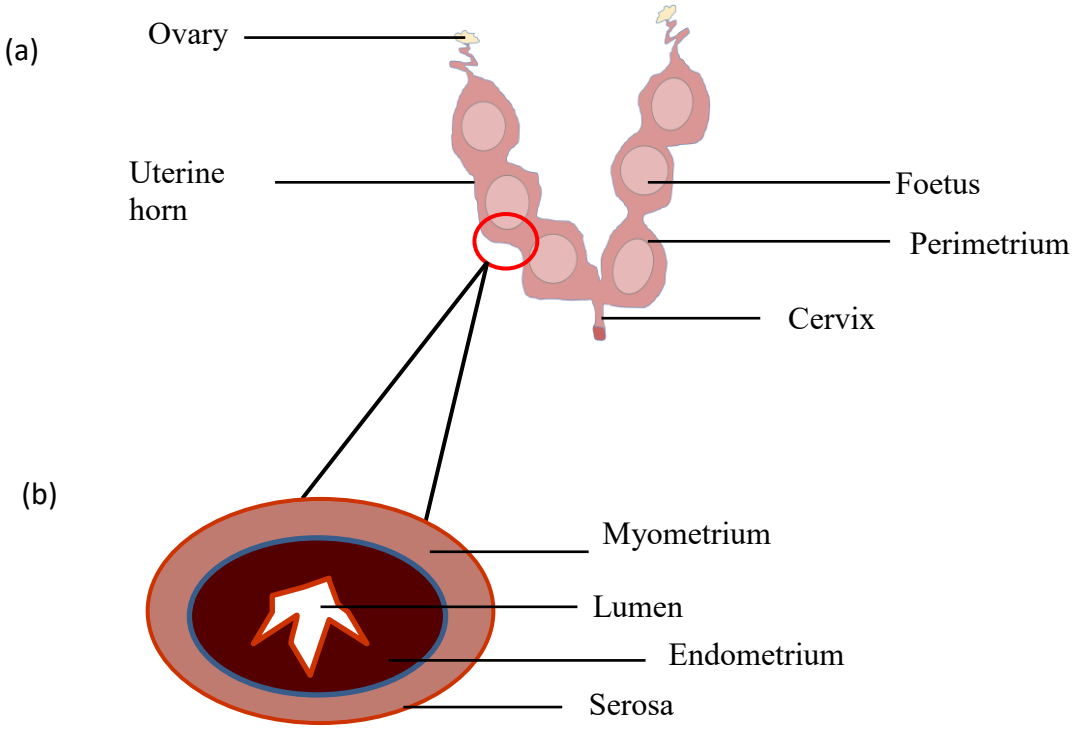


Figure 1. 2: The Anatomy of the mouse uterus

(a) An illustration of the mouse duplex uterus, showing each horn with its respective ovary, uterine tube, and cervix. (b) Schematic of the uterine horns showing the lumen and the myometrium sandwiched between the endometrium and serosa.

1.2 Mouse pregnancy and parturition

Pregnancy is the process of carrying one or more developing embryo or spermatozoa forming a zygote and ends once the foetus and the placenta are expelled from the uterus (Mahendroo, 2012). The length of gestation, physiological and molecular events leading to foetal development, maturation and subsequently labour vary between species. Mouse pregnancy is considered term at approximately day 19. Pregnancy in humans is maintained by P4 synthesised in the corpus luteum during the first seven weeks of pregnancy, a role which is taken over by the placenta from week 12 to term (Mesiano, 2009). In rodents, pregnancy is maintained by the corpora lutea, such that the removal of the ovaries results in labour and delivery of the pups (Maeda et al., 2000). Parturition is a complex physiological process whose fundamental determinants have remained elusive. Parturition is initiated in most non-primate mammals, including mice, through a decrease in circulating progesterone caused by elevated prostaglandins. The process of parturition is divided into four phases. Phase 0 is the quiescent phase in which the uterus is kept in a state of quiescence due to the repression of uterotonic signals as well as the unresponsiveness of the uterus to uterotonic stimulation (Girotti and Zingg, 2003). This phase constitute the longest phase of pregnancy. This is followed by phase 1 also known as the activation phase which is characterised by an increase in capacity to respond efficiently to uterotonic signals. This phase involves possible changes in oestrogen levels. Phase 2 is the stimulation phase and is characterised by increase in the activity of uterotonins: oxytocin and prostaglandins, and the uterotonic signals result in uterine contractions. Phase 3 is the involution phase of parturition and follows delivery of foetus and placenta as well as return of the uterus back to normal dimensions of non-pregnant state.

Parturition in mouse is preceded by a sharp decline in the levels of circulating progesterone, a phenomenon that is not observed in human parturition. Labour in humans occurs in the presence of high, and sometimes increasing, levels of progesterone (Zakar and Hertelendy, 2007). The role of Prostaglandins F2α (PGF2α) in the initiation of labour in mice was confirmed in studies of genetically altered

mice. A study showed that labour could not be initiated in FP knock-out mice, $FP^{-/-}$, as luteolysis and progesterone withdrawal did not occur (Sugimoto et al. 1997). In addition to the maintenance of high circulating levels of progesterone in $FP^{-/-}$ mice, OTR expression was not upregulated in uterine tissues as is consistent with the onset of labour. In mice lacking polylactide (PLA2), and mice lacking the constitutive COX-1 enzyme, progesterone levels remained elevated while labour was delayed, suggesting that PLA2, COX-1, PGF2α, and FP are required for luteolysis and the systemic withdrawal of progesterone in mice at term (Ratajczak and Muglia, 2008, Sugimoto et al., 2015).

1.3 The Myometrium

1.3.1 The Structure of the Myometrium

The uterus of humans and mice is a myogenic organ (Wray, 1993), spontaneously contracting *in vivo* and *in vitro* without neural or hormonal stimuli. These contractions are produced by the myometrium which consists overwhelmingly of uterine smooth muscle cells. Uterine smooth muscle has a phasic contractility pattern, maintaining its resting tone with discrete, intermittent contractions, of varying frequency, amplitude and duration, depending upon gestational and hormonal conditions (Aguilar and Mitchell, 2010).

In human myometrium, four distinct layers have been identified: the fasciculus layer (consisting of myocyte and connective tissue), the cylindrical bundles (consisting of densely packed myocytes), sheet-like bundle (consisting of densely packed sheet of myocytes), fibre bundle (consisting of loosely packed and few myocytes), and communicating bridge (consisting of large bridges adjacent to fascicule) (Young and Hession, 1999). In mouse myometrium however, there are only two distinct layers: the outer longitudinal and inner circular smooth muscle layers.

1.3.2 Myometrial contractile machinery

Myometrial cells are long and spindle-shaped, which is typical of most visceral smooth muscle. These cells vary in length (300 μm to 600 μm) and diameter (5 μm to 10 μm). These cells are densely filled with muscle fibres (myofilaments) which occupy 80-90% of the total cell volume, with the remaining 10-20% taken up by the nucleus, sarcoplasmic reticulum and mitochondria (Aguilar and Mitchell, 2010).

The myometrium has thin and thick filaments with the main components of these filaments being actin and myosin respectively. In the uterine smooth muscle, there is approximately 6-fold more actin than myosin (Word et al., 1993).

Actin filaments are circular, soluble globular protein measuring 6-8 nm in diameter. There are six isoforms of actin which are expressed from different genes (Aguilar and Mitchell, 2010), with the main actin isoforms (α, β and γ type) expressed in myometrial cells (Taggart and Morgan, 2007). The main roles of the different actin

isoforms are yet to be established, however, it is suggested that α- and β-actin are invariant with respect to total protein during pregnancy whereas γ-actin is suggested to exhibit an increased expression and altered localization towards term (Taggart and Morgan, 2007). Smooth muscle actin has been suggested to exist as part of both a contractile domain directly involved in force-generating events and a cytoskeletal domain important for structural integrity.

Myosin filaments, which measure 15-18 nm in diameter are the prototype molecular motor (Taggart and Morgan, 2007). This protein converts chemical energy in the form of ATP to mechanical energy, thus generating force and movement. The size of myosin in different tissues varies, but in smooth muscle, myosin is 470kDa in size, with two globular head groups joined by 150nm long tail. Associated with each head are 20 and 17kDa light chains. Contraction and relaxation are regulated by the phosphorylation of serine 19 on the 20kDa chains (Word et al., 1993). The actin and myosin filaments run in parallel and in the longitudinal dimension of the cell. Smooth muscles also contain intermediate filaments, so called because of their diameter (10 nm). These filaments are composed predominantly of desmin (Leoni et al., 1990).

1.4 Gap junctions

In various tissues, it is well established that gap junctions drive the cell-to-cell communication. The gap junction proteins are regulated primarily through endocrine mechanisms and the protein density is up-regulated by oestrogen and prostaglandins, and down-regulated by progesterone. There is also evidence that an increase in Ca^{2+} and a decrease in pH can reduce the permeability of gap junctions (Spray and Bennett, 1985, Skerrett and Williams, 2017). In the myometrium, gap junctions play a leading role in the transition from a quiescent to an excitable state. In the final stages of gestation, the gap junction content in the myometrium has been shown to increase (Garfield et al., 1977) and the changes or increase in gap junction content may be relative to the changes in hormonal levels (Garfield et al., 1979).

In non-pregnant myometrium and early pregnancy, only few gap junctions have been shown between the cells (Miller et al., 1989). There is a drastic increase in the number of gap junctions in the days preceding labour. These values reach a peak at term and delivery (Miller et al., 1989). During normal delivery, an individual cell will have around 1000 gap junctions which increase in diameter to around 250 nm (Garfield et al., 1977). This is then followed by a dramatic down-regulation of gap junctions within 24 hours of delivery.

The channels enable electrical signals to be transmitted throughout the myometrium and so allow the uterus to synchronise contractions in labour. This synchrony of contractions occurs through transmission of electrical activity via connected myofibrils to muscle fibres. The activated myocytes produce prostaglandin, and cause depolarisation of surrounding myocytes. This activity continues as more myocytes are activated leading to contraction (Smith et al., 2007). The permeability of the junctions is up-regulated by oxytocin, and downregulated by relaxin. While an increase in the gap junction density increases tissue conductance, myogenic mechanisms are required to initiate activity within the cell.

The regulation of gap junction coupling is thought to determine uterine electrical activity in two ways. First, preterm, uterine quiescence is enforced by limiting the flow of excitation from cell to cell. Second, during labour, an increased amount of coupling is needed for robust contractile activity (Miyoshi et al., 1996). There is no question that more gap junctions will enhance cell-to-cell communication; however, there is no relevance if the new gap junctions do not open or if their contribution to improved coupling is not significant (Miyoshi et al., 1996). Increased propagation might be demonstrated as an increase in either the velocity of propagation or the distance over which propagation occurs. Miller et al. (1989) studied the propagation of spontaneous activity in muscle strips *in vitro* and found both propagation velocities and distances at term to be about 2 or 3 times the preterm values. Their findings show a net increase in propagation at term, the expected effect of increased junctional conductance (Miyoshi et al., 1996).

1.5 Myometrium cell excitability

The ability of the uterus to contract is made possible by the underlying electrical activity of the myometrial cells (Wolfs and van Leeuwen, 1979).

It is established that excitation of the myometrial cells relies on the movement of K^+, Na^+, Cl^- and Ca^{2+} ions, through their respective ionic channels (Fatt and Ginsborg, 1958). Although these ions determine the resting membrane potential of the cell resulting in the generation of action potential, the resting membrane potential is predominantly set by K^+. An influx of Ca^{2+} is driven by spontaneous changes in membrane potential (Wray, 1993), depolarising the cell and allowing smooth muscle contraction whereas an efflux of potassium leads to repolarisation that causes cell relaxation. These phenomena indicate that the excitability of the uterus is modulated through ionic transport, and relies on the gating properties of ion channels.

1.5.1 Electrical activity and Membrane potential

The uterus is a myogenic organ and an excitable tissue, spontaneously contracting without the need for an external stimulus. It fires an action potential and has a resting membrane potential (Wray et al., 2015). The initiation as well as coordination of its activity is characterised by cycles of depolarisation and repolarisation that occur within the plasma membrane. These changes constitute action potentials.

Resting membrane potential is where minimal or no change in membrane potential occurs. The resting membrane potential in the uterus has been measured to be between - 35mV and - 80mV. The value varies depending on the gestational state and muscle layer (Parkington et al., 1999). This value may also be different between species. In human, the resting membrane potential at term is reported to be approximately -55mV (Parkington et al., 1999). This becomes more negative towards mid-pregnancy (- 60mV) and increases near term and parturition to - 45 mV (Kuriyama and Suzuki, 1976, Parkington et al., 1999) and these values are similar to that in pregnant rat (Kuriyama and Suzuki, 1976).

The excitability of the myometrium is controlled by the movements of Na^+, K^+, Cl^- and Ca^{2+} ions between the intracellular and extracellular spaces (Aguilar and Mitchell, 2010). The myometrial plasma membrane is known to be more permeable to the K^+ ions. The ionic gradient is maintained such that the K^+ ion content within the cell is high, and Ca^{2+} level is low (Kao et al., 1989). While the K^+ is concentrated within the cytoplasm, Na^+, Cl^- and Ca^{2+} are found to be concentrated outside the uterine myocytes (Sanborn, 1995).

Upon depolarisation of the plasma membrane, there is an influx of Ca^{2+} which occurs as a result of voltage gated calcium channel (VGCC) activation. Repolarisation is caused by an outward flow of potassium ions (Figure 1.3). Depolarisation of the surface membrane is associated with pacemaker activity and the presence of pacemaker cells in the myometrium remains to be elucidated (Shmygol et al., 2007). Interstitial cells of Cajal (ICC) have been located in other smooth muscles such as urinary bladder and gastrointestinal smooth muscle. These ICC-like cells also known

as telocytes have also been found in myometrial smooth muscle (Duquette et al., 2005). Uterine telocytes are also assumed to play a pacemaker role in the myometrium (Othman et al., 2016); however, there remains a debate whether these cells perform this function. (Roatesi et al., 2015) suggests that based on available data, the pacemaking role of ICC is not required in the uterus (Campeanu et al., 2014) but is needed in organs like gut (Carmona et al., 2011), vasculature (Cantarero et al., 2011) and urinary tract (Gevaert et al., 2012) that require permanent peristaltic wave action to remove their content. In fact, the lack of regular slow waves of depolarisation in uterine telocytes has already been shown (Duquette et al., 2005, Cretoiu et al., 2013), supporting this point. In the pregnant uterus, the site and behaviour of pacemakers has been long researched (Rabotti and Mischi, 2015, Lammers, 2012) . It has previously been suggested that uterine contractions originate from the fundus (Buhimschi, 2009), however in the rat, pacemaker activity is suggested to occur more frequently at the ovarian end of the horn (Crane and Martin, 1991). Recent imaging studies have confirmed that in guinea pig and rat, the pacemaker area originates at the mesometrial border or near the ovarian end (Lammers et al., 2015).

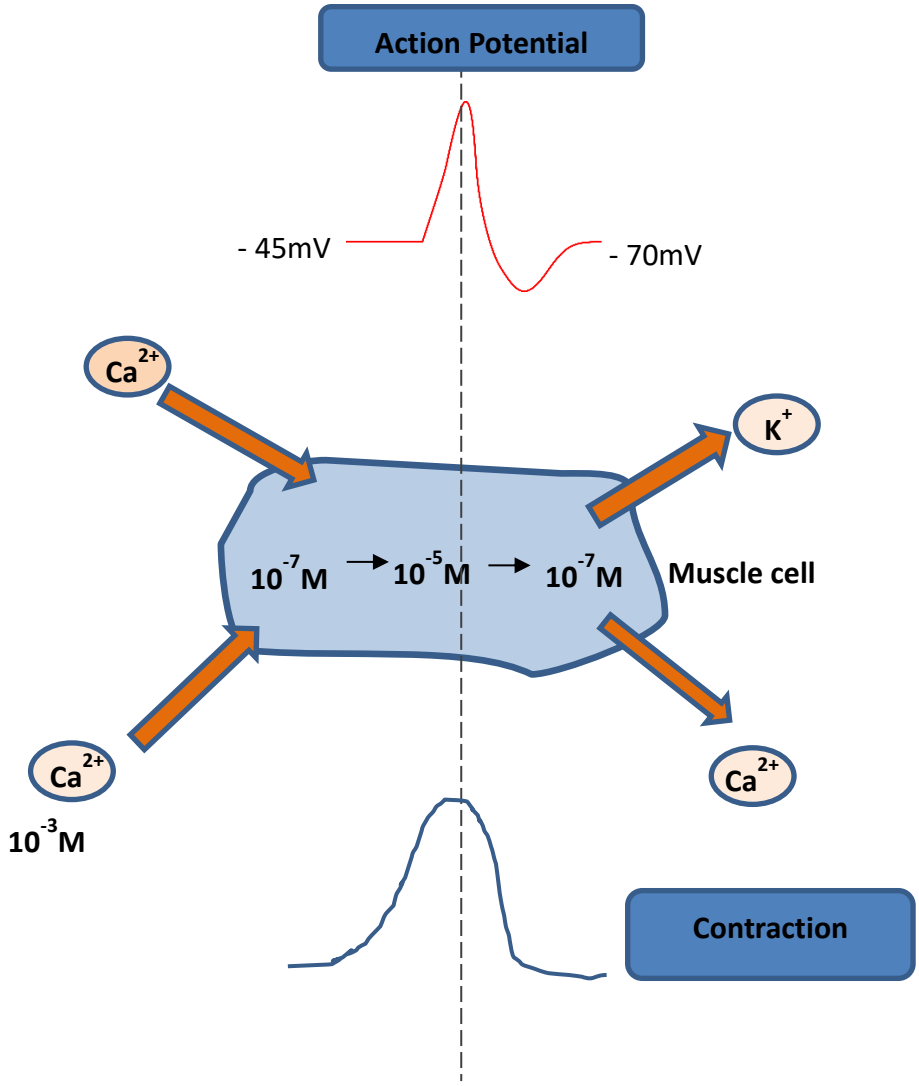


Figure 1. 3: Schematic representation showing membrane potential, calcium flux and muscle contraction

Due to ion flux across the membrane, the membrane potential gradually rises and falls but these fluctuations do not cross the threshold level. Once calcium channels open, calcium enters into the cell and depolarisation occurs, causing generation of action potential. When potassium channels open, potassium leaves the cell, causing repolarisation, the calcium channel closes and the action potential is terminated.

1.5.2 Voltage-gated calcium channels

The voltage-gated calcium channels (VGCC) are activated by changes in membrane potential. They are divided into three main types: Cav 1 (L-type), Cav 2 (P-type, N-type and R-type) and Cav 3 (T-type calcium channels) (Lipscombe et al., 2004), of which the L-type and T-type have been identified and expressed in the myometrium (Lee et al., 2009, Young et al., 1993). A full list of the subunits and their function is shown in Table 1.1. Both channels have been reported in human (Blanks et al., 2007, Parkington and Coleman, 2001), and animal (Collins et al., 2000, Ohkubo et al., 2005) myometrium.

The T-type calcium channels comprise of three main subunits: Cav 3.1, Cav 3.2, and Cav 3.3. They are thought to play a role in the generation and modulation of frequency, by producing low voltage activated currents in spontaneous uterine contractions (Lee et al., 2009). Although the T-type calcium channels have been identified in the myometrium, the L-type channels are regarded the major channel for calcium entry and excitation-contraction coupling (Lipscombe et al., 2004).

The L-type calcium channel is a hetero-oligomeric complex of protein consisting of different subunits: α1, β, γ and α2/δ (Collins et al., 2000) (Figure 1.4). The α1 subunit contains the channel pore and has the characteristic functional and pharmacological properties of calcium-channel ion permeability; voltage sensing and agonist binding, whereas the others *are* auxiliary subunits that modulate channel activity (Keef et al., 2001). These channels produce high-voltage, long lasting currents and account for the calcium current in the myometrium (Shmygol et al., 2007). They comprise of four main α subunits: Cav 1.1, Cav 1.2, Cav 1.3 and Cav 1.4.

Nifedipine, the L-type calcium channel blocker inhibits spontaneous uterine contractions, indicating that L-type calcium channel is the main source of calcium entry (Wray et al., 2003). In addition, removal of calcium from a solution bathing the uterus results in the contractions ceasing. Direct or indirect agonist stimulation could result in L-type calcium channel opening without depolarizing the membrane through guanosin-5'-triphosphate binding proteins (Wray, 1993).

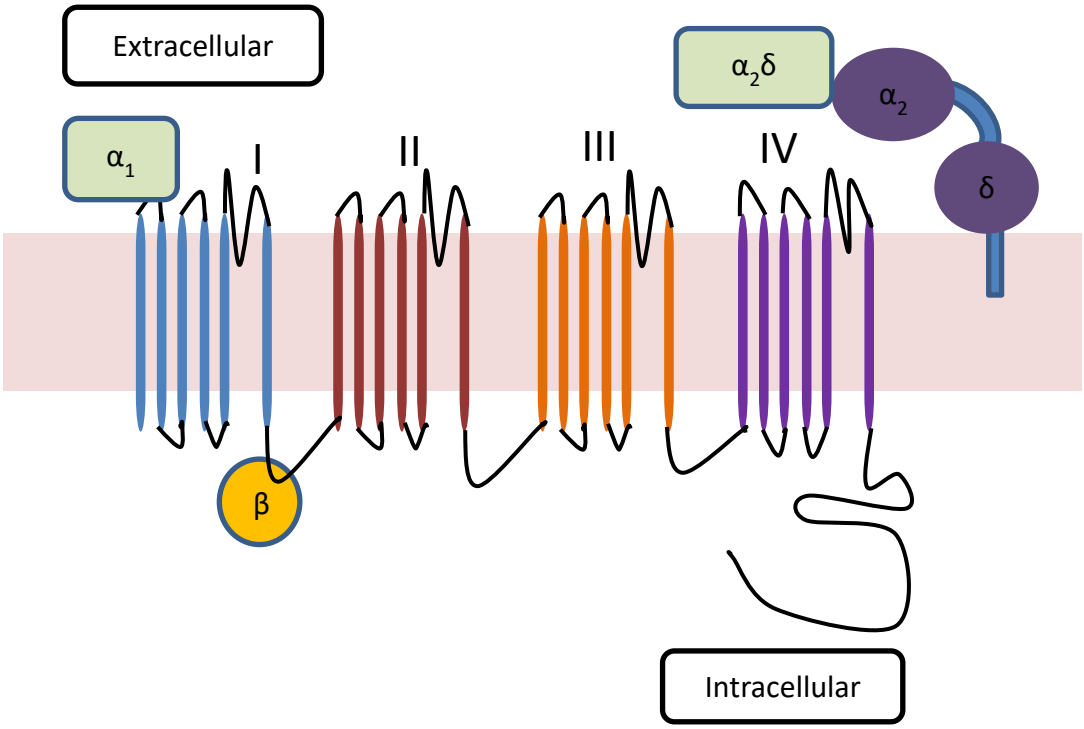


Figure 1. 4: Subunit structure of L-type voltage gated calcium channel

The α1-subunit forms the ion-conducting pore and is comprised of four repeats (I, II, III and IV) with each repeat containing six membrane-spanning domain. Adapted from Dolphin 2018.

Type	Subunits	Specific blocker	Functions
L-type	Cav 1.1	Dihydropyridine (DHP)	Excitation-contraction coupling in skeletal muscle
L-type	Cav 1.2	DHPs	Excitation-contraction coupling in cardiac and smooth muscle
L-type	Cav 1.3	DHPs	Hearing, sinoatrial node function
N-type	Cav 1.4	DHPs	Synaptic transmission in CNS, motor nerves
P/Q-type	Cav 2.1	ω-CTx-GVIA	Neurotransmitter release, Dendritic Ca^{2+} transients
R-type	Cav 2.2	ω-Agatoxin	Synaptic transmission in Peripheral nervous system (and Central nervous system especially in early development)
T-type	Cav 2.3	SNX-482	Neurotransmitter release, Dendritic Ca^{2+} transients
T-type	Cav 3.1	None	Neuronal excitability, pacemaker activity and repetitive firing
T-type	Cav 3.2		Pacemaker activity and repetitive firing
T-type	Cav 3.3		Pacemaking and neuronal excitability

Table 1. 1: Calcium channel types, subunits and physiological functions

(Dolphin, 2018)

1.5.3 Potassium channels

During pregnancy, the uterine smooth muscle changes remarkably. Although the uterus is a myogenic organ, spontaneously contractility, nevertheless, it must remain quiescent during the changes that occur during stretch resulting from pregnancy and suddenly develop powerful and organized contraction needed for labour (Smith et al., 2007). This transition in the uterus from quiescent state during pregnancy to labour at the end of gestation is thought to be propelled by changes in electrical currents within the myometrial cells (Smith et al., 2007), and potassium channels form a part of the mechanism that allows for these changes in the uterus.

In the myometrium, several types of K^+ channels have been identified including: calcium-activated potassium (K_{Ca}) channel (Perez et al., 1993), the ATP-sensitive K^+ (K_{ATP}) channel (Modzelewska et al., 1998), and voltage-gated K^+ (Kv) channels (Knock et al., 1999). The variety of K^+ channels present in the myometrium indicates the complexity of the mechanisms involved in the regulation of the muscle tone (Brainard et al., 2007).

- ***Calcium activated K^+ channels***

  In smooth muscles, there are three subclasses of K_{Ca} channels that are activated with increase in intracellular Ca^{2+} levels (Blatz and Magleby, 1987, Vergara et al., 1998). They include the large or big conductance calcium-activated K^+ (BK_{Ca}), which is the most abundant and well-studied in smooth muscle, including the myometrium; the intermediate calcium-activated K^+ (IK_{Ca}); and small conductance calcium-activated K^+ (SK_{Ca}) channels.

  The BK_{Ca} channel is shown to be the predominant K^+ channel type in non-pregnant (Pérez et al., 1993), pregnant human myometrium (Anwer et al., 1993, Khan et al., 1997) and non-pregnant rat (Song et al., 1999). In normal pregnancy, increased intracellular Ca^{2+} levels activate the BK_{Ca} channel (Khan et al., 1993, Anwer et al., 1993), which could decrease membrane excitability and maintain quiescence throughout pregnancy. Using K+ channel blockers: tetraethyl ammonium chloride, barium and 4-aminopyridine, BK_{Ca} channels are inhibited, resulting in increased force and frequency of contractions in human myometrium (Anwer et al., 1993,

Khan et al., 1998), partly suggesting that a few of BK_{Ca} channels are active at rest and may play important role in tissue function. In rat myometrium however, inhibition of BK_{Ca} channels has resulted in little or no effect in non-pregnant and pregnant contractions. The mechanisms underlying ion channel function in labour have not been fully understood. Studies in pregnant uterus have shown that BK_{Ca} channels appear to lose their Ca^{2+} and voltage dependence with the onset of labour (Khan et al., 1997, Khan et al., 1993); overall, the expression of BK_{Ca} channels is reduced with the onset of labour (Noble et al., 2009).

Physiologically, the IK_{Ca} channels play an important role in salt and fluid transport. They also maintain a negative resting membrane potential to help accumulate electrical gradients for ion transport (Jensen et al., 2001, Begenisich et al., 2004). IK_{Ca} channels have a similar structure consisting of a central pore, a calcium sensing region and 6 transmembrane spanning regions (Tharp and Bowles, 2009) and unlike the BK_{Ca} channels, they lack a voltage sensing region, therefore unaffected by membrane voltage.

The SK_{Ca} channels have been shown to be expressed in myometrium (Noble et al., 2010). They have a similar structure to voltage gated potassium channels (Maylie et al., 2004), contribute to outward current and regulate membrane potential. The SKCa channels are encoded by four genes: SK1, SK2, SK3 and SK4. They don't contain a calcium binding motif to allow direct interaction with calcium, however the proximal c terminal domain interacts with calmodulin (Maylie et al., 2004). SK1-3 give rise to SK channels that are sensitive to the venom apamin, activated by calcium but act independent of membrane voltage while the SK4 is apamin insensitive and has greater unit conductance (Maylie et al., 2004). The SK channels play a role in the myometrium as regulators of contractions during gestation and labour (Brainard et al., 2007).

Studies have shown that the expression of SK2 can affect the synchronisation of uterine contractions by inhibiting calcium entry though L-type Calcium channels (Brown et al., 2007). In the mouse uterus, SK3 channels expression has been demonstrated to depress phasic contractions by inhibiting calcium influx. In

transgenic mice, overexpression of SK3 has been shown to delay parturition resulting from inefficient uterine contractions (Bond et al., 2000). Other studies in humans have shown that SK3 channels are downregulated in pregnancy, signifying a role in maintaining uterine quiescence (Rahbek et al., 2013, Mazzone et al., 2002).

- ***ATP-sensitive potassium (K_{ATP}) channels***

  The K_{ATP} channels were first described in cardiac myocytes following their closure in response to high intracellular ATP levels (Noma, 1983). They play a major role in coupling cellular metabolism to membrane excitability. The K_{ATP} channels belong to the class of K^+ channel known as inward rectifiers, formed from two membrane-spanning regions (M1 and M2) flanking the pore (H5) and therefore distinct from voltage-gated K+ channels (Khan et al., 2001).

  In human myometrium, the mRNA expression of Kir 6.1, Kir 6.2, SUR1 and SUR2B (isoforms of K_{ATP}) have been detected and Kir6.1/SUR2B appear to be the predominant (Curley et al., 2002).The K_{ATP} exist in the rat myometrium and is composed of Kir 6.1 and SUR2B subunits (Xu et al., 2011). In the mouse myometrium, K_{ATP} seem to play an essential role via activation of Kir 6.2 and SUR2B (Hong et al., 2016).

  The functional role of K_{ATP} channels have been assessed through use of channel agonists and antagonists. In human myometrium, K_{ATP} channel openers can inhibit spontaneous and oxytocin-stimulated contractions (Morrison et al., 1993). One K_{ATP} opener, diazoxide showed less inhibitory effect in term labouring tissue compared to non-labouring suggesting less of a role for K_{ATP} channels in labour and overlaps with the reduction in potassium channels expression, allowing for the onset of powerful contractions. On the other hand, a K_{ATP} channel blocker glibenclamide induces spontaneous uterine contractions (Bailie et al., 2002). There is evidence of varied results of K_{ATP} channel effect between pregnant and non-pregnant myometrium indicating difference in expression during gestation (Longo et al., 2003, Bailie et al., 2002). An increase in outward potassium current causes hyperpolarisation of the surface membrane and decreases excitability of the smooth muscle cells (Heaton et al., 1993).

- ***Voltage gated potassium (K^+) channels***

  Voltage-gated K^+ (K_v) channels are widely expressed in the myometrium, and play a role in maintaining resting membrane potential (Brainard et al., 2007). They are also involved in maintaining quiescence and initiation of uterine contractions (Knock et al., 1999). In response to depolarisation, K^+ efflux occurs via these channels, which induces repolarisation of the membrane (Figure 1.3).

  There are four main subtypes of Kv channels: K_v1 (Shaker), K_v2 (Shab), K_v3 (Shaw) and K_v4 (Shal), and the K_v4 subtype plays the most important role in myometrium during pregnancy (Knock et al., 1999) and are the most subtype identified in human myometrium (Brainard et al., 2007) . The expression of $K_V4.1$ and 4.3 has been shown to decrease throughout gestation while the $K_v4.2$ appears to increase before parturition (Suzuki and Takimoto, 2005). The decrease of $K_v4.1$ and 4.3 throughout gestation possibly suggests the involvement of hormones in regulating the channels in pregnancy. Another study in ovariectomised rats revealed that oestrogen down-regulated membrane localisation, protein and current expression of $K_v4.3$ channels (Song et al., 2001). Although Kv channels are thought to play a major role in reversing membrane depolarisation, during an action potential (), it is shown that Kv channels can also regulate resting membrane potential in smooth muscles like airway (Fleischmann et al., 1993) and arteries (Ishikawa et al., 1997, Gurney et al., 2003)

  In mouse myometrium, the expression of Kv channel subtypes have been previously examined (Smith et al., 2007, Shi et al., 2016). Smith et al., who examined Kv expression across gestational states of mice, demonstrated differences in the expression levels of some Kv subunits. They showed a higher Kv1 expression in pregnant myometrium than non-pregnant. They also showed a higher Kv4.3 expression in pregnant compared to non-pregnant tissues; however there was a profound reduction close to term. Similar reduction in Kv4.3 expression was observed in rat uterus (Song et al., 2001). Their findings suggest that besides regulating uterine contractions, Kv channels may have a role in functional changes that occur in pregnant myometrium.

- ***Other K channels***

Although not as well studied, there is evidence for two-pore domain acid sensing K^+ (TASK) channels in the myometrium. It is shown that the activation of TASK-2 channels might play a major role in uterine relaxation (Kyeong et al., 2016). TASK-2 channels are sensitive to changes in extracellular pH (Duprat et al., 1997) and can be inhibited by several pharmacological inhibitors (O'Connell et al., 2002). In the presence of stretch-activated channels, TASK-2 inhibitors resulted in strong longitudinal mouse myometrium (Kyeong et al., 2016). Similar result was obtained in uterine circular mouse myometrium when using TASK-2 channel inhibitors (Hong et al., 2013). In comparison to non-pregnant myometrium, pregnant myometrium showed increased expression of TASK-2 channels (Kyeong et al., 2016) and this increased expression of TASK-2 channels might explain how the uterus accommodates the growing foetus during pregnancy. It is suggested that targeting these channels might be a means to preventing preterm labours.

1.5.4 Sodium (Na^+) channels

As well as Ca^{2+} and K^+, Na^+ is also considered an important ion in regulating myometrial cell excitability (Anderson et al., 1971). Although not fully characterised, two voltage-gated Na^+ channels: $Na_v2.1$, $Na_v2.3$ have been reported to be expressed in human and mouse myometrium (George et al., 1992, Phillippe, 1998). Sodium channels generate fast inward current resulting in membrane depolarization. In ovariectomised and oestrogen-treated rats, Anderson *et al* showed that Na^+ and Ca^{2+} are essential to produce action potentials in myometrial tissue (Anderson et al., 1971). In human pregnant myometrium, similar result was obtained (Inoue et al 1990). In pregnant rats, Kao and McCullough showed that Na^+ is the main carrier of the early inward current in day 18-21 myometrium (Kao and McCullough, 1975). A more recent study in non-pregnant rat suggest that the activation of Na^+ channels cause opening of L-type calcium channels, resulting in Ca^{2+} influx and initiation of contraction (Seda et al., 2007).

Some animal studies have shown an increase in Na^+ current during pregnancy. Using electrophysiological experiment, (Inoue and Sperelakis, 1991) concluded that

Na^+ current density increases towards term partly due to an increased number of cells expressing Na^+ channels. They also detected the fast inactivating and tetodoxin-sensitive Na^+ channels. Similar findings were observed in another study (Ohya and Sperelakis, 1989). These studies also suggest that, Na^+ channels may augment uterine excitability through an elevation of intracellular Ca^{2+} due to activation of Na^+/Ca^{2+} exchanger in response to an increase in transmembrane transport of Na^+ (Ohya and Sperelakis, 1989, Inoue and Sperelakis, 1991).

Another study showed in non-pregnant myocytes stimulated with oestrogen revealed the presence of Na^+ channels and faster kinetics as gestation progressed but were nearly undetectable postpartum (Yoshino et al., 1997). The authors concluded that an increased Na^+ current density and inhibition of K^+ channels expression resulted in a more excitable myometrium starting mid-gestation and promotes coordinated uterine contractions during labour. A more recent study (Reinl et al., 2018) which investigated the mRNA and protein expression of Na^+ leak channel in mouse revealed that the expression levels decrease in mid-pregnancy, increases during labour and remains high in early postpartum period. This increase of protein expression observed during labour and in postpartum is similar to what is seen with contraction-associated proteins (Miller et al., 1989). The finding by Reinl et al., 2018 supports the findings that Na^+ leak channel play an important role in uterine excitability.

1.5.5 Chloride channels

Chloride (Cl^-) channels, which are responsible for the movement of Cl^- ions across the cell membrane, are integral membrane proteins and the contribution of these channels to the regulation of myometrial tone has been acknowledged. In smooth muscles, the concentrations of intracellular Cl^- have been measured using different techniques and range from 30-50 mM (Kitamura and Yamazaki 2001). The presence of Cl^- channels have been confirmed in the following smooth muscles: vascular (Pacaud et al., 1991), portal vein (Hogg et al., 1994), mesenteric artery (Klockner, 1993), and myometrial (Adaikan and Adebiyi, 2005).

Two types of Cl^- channels have been identified in the smooth muscle: calcium-activated Cl (Cl_{Ca}) and volume-regulated chloride (Cl_{Vr}) channels (Shi et al., 2007), which could be activated by any of/ or a combination of changes in $Ca^{2+}]_i$, cAMP, pH, extracellular ligands, cell swelling. There is limited evidence to the regulation of Cl_{Vr} in the myometrium, however, it is established that, pregnancy is associated with an increase in cell volume (hypertrophy) and Cl_{Vr} may bring about this swelling.

The Cl_{Ca} channels play a role in controlling excitability in rat myocytes (Jones et al., 2004). They have also been suggested to cause membrane depolarisation via the opening of L-type calcium channels (Jones et al., 2004) and lead to calcium influx and initiate contraction. Furthermore, inactivating Cl_{Ca} channels would result in repolarisation and subsequently inhibit calcium entry and contraction. A specific Cl_{Ca} channel blocker, niflumic acid has been shown to reduce both spontaneous and oxytocin-induced contraction in pregnant rat myometrium (Adaikan and Adebiyi, 2005). These channels play a significant role in the generation of contractile tone and spontaneous contractions in many smooth muscle organs including the myometrium (Yarar et al., 2001) and recent studies have implicated their importance as the underlying pacemaker conductance in the rat uterus (Jones et al., 2004).

1.6 Regulation of intracellular calcium in uterine contractility

1.6.1 Role of calcium in uterine contractions

Uterine contractions are initiated by an increase in $[Ca^{2+}]_i$ either through entry of extracellular Ca^{2+} into the cell or via the release of $[Ca^{2+}]_i$ from the SR (Shmygol et al., 2007, Babich et al., 2004). Consequently, the increase in $[Ca^{2+}]_i$ activates a Ca^{2+} binding cytosolic protein, calmodulin (CaM) (Figure 1.5), which can bind four Ca^{2+} ions (Johnson et al., 1996). Formation of the Ca-CaM complex activates a key enzyme, myosin light chain kinase (MLCK) by binding to it. This results in a marked increase in the phosphorylation of myosin regulatory light chains (MLC-20), and subsequent cross-bridge cycling (Shojo and Kaneko, 2001). Studies show that phosphorylation of MLC-20 by MLCK is a major factor of smooth muscle contraction (Longbottom et al., 2000). Myosin light chain kinase is highly specific and phosphorylates the serine 19 on MLC-20. This initiates actin-myosin ATPase on myosin heavy chains generating mechanical energy for contraction (Kamm et al., 1989).

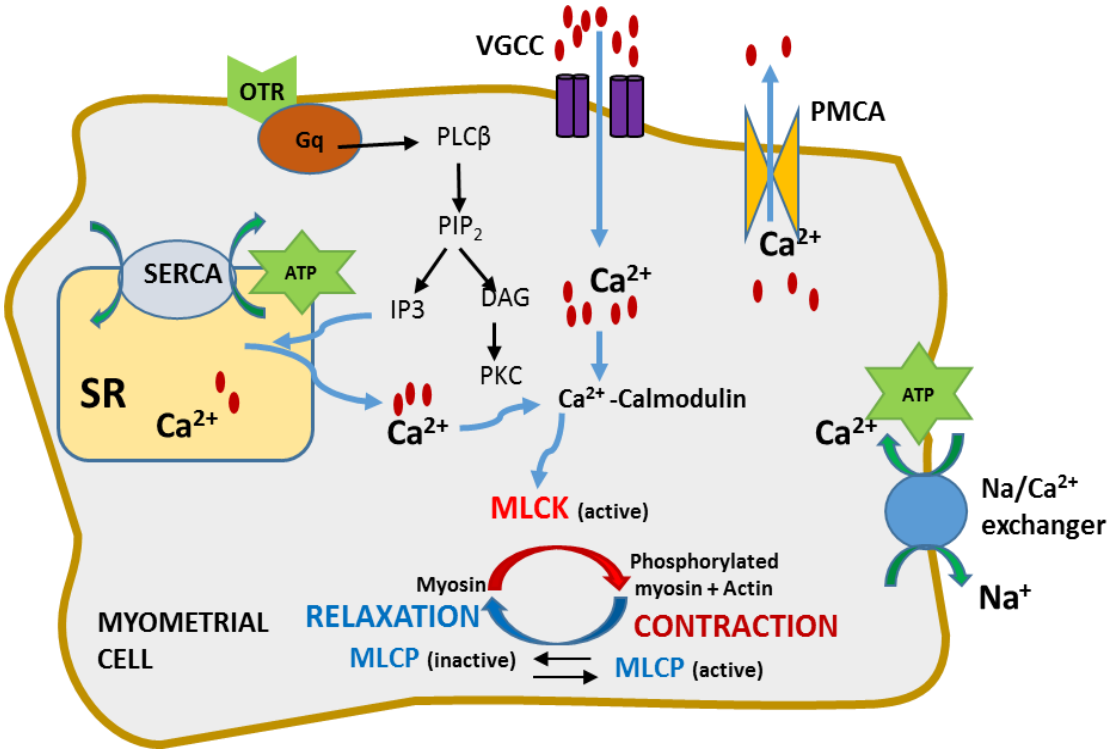


Figure 1. 5: Schematic presentation of calcium entry and initiation of contraction in uterine smooth muscle.

Agonist binds G-protein coupled receptor thereby activating G-protein and opening of receptor-operated and plasma membrane Ca^{2+} channels. In parallel, a cascade of events is turned on. The G-protein G_q stimulates PLC to cleave PIP_2 into DAG and IP_3. This induces Ca^{2+} release from internal stores resulting in a rise in $[Ca^{2+}]i$. This calcium then binds to calmodulin (CaM) forming a complex which then activates myosin light chain kinase (MLCK). Once activated MLCK then phosphorylates the myosin light chain (MLC) which then binds to actin causing contraction. Dephosphorylation of MLC-20 by Myosin Light Chain Phosphatase (MLCP) removes the phosphate group from MLC and leads to relaxation.

Calcium is removed from the cell via the use of the sodium calcium (Na^+/Ca^{2+}) exchanger or the plasma membrane calcium ATPase transporter protein (PMCA) or the Sarcoplasmic reticulum calcium ATPase transporter protein (SERCA) or a combination of these. The phosphate group is then removed from myosin by myosin light chain phosphatase (MLCP), leading to relaxation

1.6.2 Mechanism of calcium influx

Initiation of smooth muscle contractions results from a rise in intracellular calcium concentrations from 10^{-7} M to 10^{-6} M (Horowitz et al., 1996). Spontaneous contraction of the myometrium relies on the calcium influx from extracellular space (Kawarabayashi et al., 1989). The release of Ca^{2+} from intracellular stores, along with Ca^{2+} influx from the extracellular space contributes to the activation of the pathway that lead to actin – myosin cross bridge cycling and production of force in the presence of ATP (Aguilar and Mitchell, 2010). As well as via the L-type Ca channels (Taggart and Morgan, 2007), with agonist stimulation, calcium can also enter via the store-operated calcium channels (SOCCs), and via receptor-operated calcium channels (ROCCs).

Receptor-operated calcium channels (ROCCs) are a class of membrane bound channels that have a binding site for agonists such as neurotransmitter or hormones. The channels open when ligands bind to specific-associated receptors. Receptor operated currents have been reported in different types of smooth muscles (Smith et al., 2005, Thorneloe and Nelson, 2005).

There are two types of ROCCs that can be activated by a specific agonist. One that can be activated by ATP (Kuriyama et al., 1998) and the other, which opens because of G-protein coupled receptors (Inoue et al., 1987). Activation of ROCCs may cause fluctuations in membrane potential leading to activation or inhibition of VGCCs (Wray, 1993).

Calcium influx is also possible via the store operated calcium channel (SOCCs). As a result of calcium depletion in the SR, calcium can enter the cell via specific store channels found in the cell membrane (Putney Jr.* and Pedrosa Ribeiro, 2000). SOCC's are activated by a reduction in the SR calcium content, which results in calcium entry known as store operated calcium entry (SOCE). This rise in calcium availability may contribute to contractility in the myometrium (Tribe et al., 2000).

The role for SOCC in the myometrium is currently unclear, nevertheless, the underlying mechanism for SOCE are thought to have a relationship with transient receptor potential superfamily (TRP), which has been shown to be expressed in

human myometrium (Ku et al., 2006). The TRP family has been shown to be upregulated in term as well as labour suggesting a role in labour (Dalrymple et al., 2004).

1.6.3 Calcium efflux

Relaxation or termination of myometrial contractions occur when there is a decrease in intracellular Ca^{2+} levels within the cell. This removal occurs via the SR or plasma membrane, and is achieved via two mechanisms: the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^{+} /Ca^{2+} exchanger (Figure 1.5).

The PMCA works to maintain resting Ca^{2+} levels and is described as a high affinity system tuned to keeping Ca^{2+} low (Matthew et al., 2004a) are activated by calmodulin binding, which removes auto-inhibition and increases its affinity for Ca^{2+} (Marin et al., 1999). The Na^{+} /Ca^{2+} exchanger extrudes one Ca^{2+} for three Na^{+} ions, which results in direct effect on excitability. It utilises the energy stored in the Na^{+} gradient provided by Na/K-ATPase to allow the efflux of calcium (Floyd et al., 2017). The movement of calcium can be reversed if the gradient is also reversed, but this is unlikely during normal physiological conditions. Within uterine tissues and cells, the Na^{+} /Ca^{2+} exchanger exudes 30% while PMCA exudes 70% of the calcium that enters the cell (Shmigol et al., 1998, Shmigol et al., 1999).

Biochemically, relaxation of the myometrium follows a reversal of the Ca^{2+}–CaM MLCK pathway. Dephosphorylation of MLCs occurs by myosin light chain phosphatase (MLCP), which can also be activated by small G-protein coupled protein kinase cascade (Kimura et al., 1996). This causes a dissociation of Ca^{2+} from CaM and inactivation of MLCK (Wray et al., 2003, Wray et al., 2001).

1.6.4 Regulation of $[Ca^{2+}]_i$ by the sarcoplasmic reticulum (SR)

The uterus consists of an extensive SR, the internal store, which increases in pregnancy. The sarcoplasmic reticulum's Ca^{2+} -ATPase (SERCA) is a pump found on the membrane of SR. It transports Ca^{2+} from the cytoplasm to the lumen of the SR against the calcium concentration gradient. Active transport using the energy from ATP hydrolysis is required. Calcium release from the SR occurs via two pathways:

Agonist or inositol triphosphate (IP_3) induced Ca^{2+} release (IICR) and Ca^{2+}-induced Ca^{2+} release (CICR) via Ca^{2+} release channels (ryanodine receptors) on the SR gated by Ca^{2+} (Pehlivanoğlu et al., 2013).

The myometrium is stimulated by a variety of agonists whose receptors are coupled to G-protein. This leads to the activation of phospholipase C (PLC) and generation of the messengers IP_3 and DAG. IP_3 causes release of calcium by binding to the IP3R on the SR (Bultynck et al., 2003), activating the release of calcium from the SR into the cytoplasm thereby increasing the $[Ca^{2+}]i$. Ryanodine receptors (RyRs) sensitive to ryanodine, are activated by an increase in the local $[Ca^{2+}]$ and inhibited by phosphorylation by PKC (Bonev et al., 1997). It is mainly gated by calcium (Ca^{2+}), as cytoplasmic calcium activates RyR channels; a process known as calcium-induced calcium release (CICR). RyR receptors do not play a major role on uterine function (Noble et al., 2009), as they are non-functional.

1.6.4 Modulation of force

The transformation of uterine activity from its state of quiescence to being active is regulated by a complex network of interactions (Kota et al., 2013). This interaction could be hormonal resulting in changes in force, frequency and duration of contractions. Oxytocin, prostaglandin, vasopressin and oestrogen can stimulate while progesterone, Nitric oxide (NO) and H_2S and relaxin result in relaxation of uterine contractions.

Before the onset of labour, there are two changes that must occur: (1) the uterus must be converted from quiescence to active with coordinated contractions, and (2), the cervix must dilate, thereby allowing the passage of the foetus from the uterus (Kota et al., 2013). These changes (Figure 1.4) are accompanied by shift from progesterone to oestrogen dominance, regulation of myometrial oxytocin receptor, leading to increased sensitivity to oxytocin, increased prostaglandin synthesis, increased gap junction formation, decreased nitric oxide (NO) activity and increased influx of calcium into myometrial cells (Kota et al., 2013, Garfield et al., 1998).

At the level of plasma membrane, a number of G-protein coupled receptor can modulate the force of uterine activity. Upon G-protein coupling, activation of

different receptors would lead to stimulation of calcium entry thus initiating contraction. For instance, oxytocin and prostaglandin receptors are known to couple to the G-protein family. These two receptors are discussed further within the hormones section.

1.6.5 Effect of pH change on myometrial contraction

Changes in intracellular pH (pHi) within the uterus have been shown to cause large changes in producing force (Parratt et al., 1995, Taggart and Wray, 1995). Due to lactate production and ATP hydrolysis, the change in pHi with contractile activity may be anticipated (Taggart and Wray, 1995). Moreover, phasic contractions have been shown to be associated with acidification (Naderali et al., 1997). The first measurement of pHi in the uterus was conducted in rats using ^{31}P NMR spectroscopy (Dawson and Wray, 1985). They recorded pHi to be approximately 7.1 for non-pregnant and pregnant rat. Similar measurement (pHi 7.1-7.2) was found in human myometrium (Parratt et al., 1994).

The addition of an acid or base to contracting myometrium results in a change in contractility (Pierce et al., 2003). This addition results in a change in extracellular pH (pHo) and studies have shown that a change in the pHo may result in alteration of pHi thereby affect cell function (Wray, 1993, Austin and Wray, 1995). In human and rat myometrium, pHi has been shown to decrease while alkalinisation increase spontaneous contraction (Phoenix and Wray, 1993).

An increase in frequency of contractions has been observed with intracellular alkalinisation (Heaton et al., 1992) while intracellular acidification has been shown to inhibit uterine contractions (Taggart and Wray, 1993). Although there is evidence showing the effect of pH on calcium and force production in human and rat myometrium, not much is known about the effect of pH alteration in mouse myometrium.

1.7 Hormones in pregnancy

A complex cascade of autocrine and paracrine events involving mother, foetus, and the placenta controls pregnancy and labour. Two main changes prerequisite for labour are (i) change from the quiescent state to an actively and rhythmically contracting state and (ii) change of the cervix from a rigid structure to a soft one, known as cervical ripening (Castracane, 2000, Snegovskikh et al., 2006, Weiss, 2000). In order to achieve a full gestational length, a fine balance of these two events is required. Interactive mechanisms such as increased oxytocin sensitivity, gap junction formation, increase in the prostaglandins, progesterone withdrawal, dominance of oestrogen over progesterone in the uterus, and relaxin action can shift this balance towards contractility (Rivera et al., 1990, Holt et al., 2011, Vidaeff et al., 2008). The roles of different hormones in pregnancy and labour are discussed below.

1.7.1 Oxytocin

It is a small nona-peptide hormone secreted by the posterior pituitary gland and synthesised by the hypothalamus. Oxytocin binds to a G-protein coupled receptor expressed in myometrium (Kimura et al., 1992). Activation of the oxytocin receptor stimulates G-proteins of the Gαq family. This results in the activation of phospholipase-C β (PLCβ). The PLC-catalysed hydrolysis of phosphatidylinositol biphosphate (PIP_2) leads to the formation of two messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its specific receptor on the SR, thereby stimulating Ca^{2+} release, therefore leading to further Ca^{2+} influx. The DAG stimulates protein kinase C (PKC), but its role in myometrial contractility is unclear (Arrowsmith and Wray, 2014).

The effects of oxytocin are mediated by the expression of oxytocin receptors (OTR). The OTR belong to the heterotrimeric G-protein coupled receptor family. Oxytocin receptors are abundant in pregnant uterine smooth muscles (Sanborn, 1995, Y Ku et al., 1995), with its concentration increasing towards the end of gestation (Fuchs et al., 1984), indicating oxytocin has increased potency as pregnancy progresses. This marked increase of OTRs toward term is the most consistent finding in parturition

literature (Arthur et al., 2007). Oxytocin stimulates uterine contractions via two pathways. Directly, oxytocin stimulates myometrial contractions via the PLC, resulting in the release of Ca^{2+} from intracellular stores (Rivera et al., 1990). Indirectly, oxytocin acts by stimulating the release of prostaglandin E2 (PGE2) and F2α. Prostaglandin in turn stimulates contractions. Both OTR and PKC activate the mitogen-activated protein kinase (MAPK) pathway which leads to prostaglandin production and therefore enhanced contractile activity (Molnar et al., 1999, Strakova et al., 1998).

1.7.2 Prostaglandins

Prostaglandins (PG) are bioactive lipids, synthesised within the amnion and chorion membranes as well as the decidua. They are derived from arachidonic acid (AA) that is converted to prostaglandin by the prostaglandin synthase enzyme. In the myometrium, there are two main stimulating PGs: $PGF_{2\alpha}$ and PGE which are reported to increase in a time-dependent manner during the later stage of gestation. Active spontaneous labour is associated with an increase in PGE2 and $PGF_{2\alpha}$ leading to biochemical pathway similar to acute inflammatory response (Brown et al., 1998).

There are four PGE2 receptors subtypes: EP_1, EP_2, EP_3 and EP_4 which are all expressed in the myometrium during pregnancy (Astle et al., 2005). When PGE2 binds to either EP_1 or EP_3 receptors, it results in contraction and potentiating inflammation while the binding of PGE2 to receptors EP_2 and/ or E_{P4} would result in relaxation and suppressing inflammation (Brown et al., 1998). This suggests the role of PGE2 in maintaining quiescence.

Although prostaglandin's role in the regulation of parturition is well known, the underlying mechanism of action of prostaglandins bringing about these uterine responses is still poorly understood (Hertelendy and Zakar, 2004).

- ***Interaction between prostaglandin and oxytocin in myometrium***

  The first study supporting the interaction between PG and oxytocin in the myometrium was by Vane and Williams (Vane and Williams, 1973). They observed that PG inhibitors, indomethacin and meclofenamate reduced oxytocin-induced

contractions in isolated rat uterine strips. Other studies have shown that blocking PG biosynthesis, inhibited uterine stimulation in response to oxytocin (Garrioch, 1978, Chan, 1983). (Wilson et al., 1988) reported that oxytocin's ability to release arachidonic acids from decidua cells from women in labour is significantly greater than in non-labouring or non-pregnant women. This suggests that oxytocin activates phospholipases, enhancing PG biosynthesis thereby leading to dual stimulatory effect from PG and oxytocin.

1.7.3 Progesterone

Progesterone is a C-21 steroid hormone that plays a crucial role in pregnancy. Circulating progesterone in the system maintains uterine quiescence throughout gestation (Mesiano, 2007). In human, the corpus luteum is the main source of progesterone at the beginning of pregnancy (Garfield and Yallampalli, 1994), however this is changed to after a few weeks of pregnancy as the placenta takes over the production of progesterone to become the main source of progesterone in pregnancy and labour (Chwalisz and Garfield, 1994, Brown et al., 2004). Progesterone downregulates prostaglandin production, oxytocin receptor expression and inhibits calcium channel formation, in support of uterine quiescence (Fuchs et al., 1984, Garfield et al., 1998).

With the onset of labour, there is a drop in circulating progesterone in mice (Sugimoto et al., 1997), but not in human (Challis et al., 2000) labour. In species like mice which depend on corpus luteum as the main source of progesterone, there is a withdrawal of progesterone before parturition and it is due to the death of corpus which is mediated by prostaglandin F2α. In mice, ovariectomy produced induction of preterm labour, whereas administration of progesterone delayed parturition (Skarnes and Harper, 1972). Genetically altered mice have been used to study mechanisms for declining serum progesterone levels in mice. However, FP receptor knockout mice did not deliver their pups at term as a result of functioning corpus luteum and, hence, absence of progesterone withdrawal (Sugimoto et al., 1997). In human, the corpus luteum is also the main source of progesterone up to the 7th week of pregnancy. The role of progesterone in early human pregnancy is similar to

that of laboratory rodents, however, after the “luteo-placental shift,” when the developing placenta becomes the principal site of progesterone synthesis, the regulatory pathways diverge (Ratajczak and Muglia, 2008).

1.7.4 Oestrogen

Oestrogen plays an important role in uterine changes of early gestation. In mice, during the first 2 days of gestation, phase I oestrogen secretion occurs. During this stage, pre-ovulatory oestrogen stimulates proliferation of the luminal and glandular epithelial cells (Robertshaw et al., 2016). At day 3 of gestation, corpora luteal are formed, and progesterone secretion stimulates stromal cell proliferation. This becomes further potentiated by preimplantation oestrogen (phase II oestrogen secretion) on day 4, the day of implantation (Huet-Hudson et al., 1989). This phase of oestrogen secretion before implantation ceases epithelial cell proliferation and allows for differentiation to occur (Tan et al., 1999). During the remodelling of the uterine epithelium, the epithelial cells lose polarity through downregulation of the cell-to-cell adhesion molecule E-cadherin (Daikoku et al., 2011, Li et al., 2015). Increased endometrial capillary permeability at the location of the blastocyst is also exhibited, contributing to implantation (Matsumoto et al., 2002).

1.7.4 Relaxin

Relaxin is a potent vasodilator, relevant in regulating haemodynamics in pregnant and non-pregnant states (Conrad et al., 2004). Relaxin may play roles within the uterus that are important for implantation, placentation and pregnancy maintenance (Napso et al., 2018). In mice and other rodents, the concentration of circulating relaxin peaks towards term (Marshall et al., 2018). In pregnant mice, relaxin is suggested to play a role in renal function during pregnancy as its deficiency leads to proteinuria (O'Sullivan et al., 2017). Studies also show stiffer uterine vessels during pregnancy with relaxin-deficient mice (Gooi et al., 2013). It is further suggested that relaxin may be promoting blood flow to the pregnant uterus (Marshall et al., 2018).

In early mouse pregnancy, relaxin modulates the uterine expression of genes involved in steroid hormone action and remodelling (Marshall et al., 2016). Studies

in mice with deficiency in relaxin signalling showed that obstructed deliveries occur at a higher rate and this was due to poor cervix maturation (Krajnc-Franken et al., 2004, Kaftanovskaya et al., 2015). In summary, relaxin plays a key role in the initial changes at the start of pregnancy, as well as preparing the mother for lactation (Napso et al., 2018).

1.7.5 NO and H_2S

H_2S, the third endogenous gaseous transmitter is a biological gas endogenously produced from L-cysteine in a reaction catalysed by two enzymes: cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE) (Hosoki et al., 1997, Li et al., 2011) and both CBS and CSE have been identified in the placenta and myometrium (Holwerda et al., 2012, Hu et al., 2016). H_2S is involved in many physiological and pathophysiological processes including vasodilation (Skovgaard et al., 2011) angiogenesis (Papapetropoulos et al., 2009), and inflammation (Wallace et al., 2012). In several tissues, the effects of H_2S on smooth muscle contractile activity have been examined and it is generally associated with a reduction in contractile activity.

In both rat and human myometrium, it has been shown that H_2S is produced, with expression of CSE and CBS and that hypoxia increases H_2S production (Patel et al., 2009, You et al., 2011). You et al also showed that endogenous H_2S produced by CSE and CBS suppresses the spontaneous contractility of pregnant human myometrial strips and that the expression of both enzymes as well H_2S production decreased with the onset of labour (You et al., 2011). Their finding suggests that endogenous H_2S is involved in the initiation and progress of labour in women. A more recent study by the same group showed that endogenous H_2S produced locally play a critical role in the maintenance of uterine quiescence, and this effect is associated with suppression of inflammation in uterus (You et al., 2017).

Nitric oxide (NO) is a reactive gas with a very short physiological half-life. It is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS) (Ledingham et al., 2000). Studies have demonstrated the ability of NO donors to decrease spontaneous and induced myometrial contraction in a cyclic guanosine

monophosphate (cGMP)-dependent and a cGMP-independent manner (Kuenzli et al., 1996, Yallampalli and Garfield, 1993). NO regulates smooth muscle cell contractility and spontaneous contraction during the oestrous cycle (Izumi and Garfield, 1995). The activity of NOS in maternal tissues increases early in pregnancy. It has been demonstrated an increase in expression of inducible NOS mRNA in rat uteri and a small increase in expression of endothelial NOS and neuronal NOS mRNA during late pregnancy (Ali et al., 1997). Immunohistochemistry study show that there is expression of inducible NOS and endothelial NOS in myometrium on days 17–18 of pregnancy and that expression decreased at term in rats in labour (Riemer et al., 1997). It is suggested that the increase in inducible NOS expression in may be important in the maintenance of uterine quiescence during pregnancy (Kaya et al., 1998, Izumi and Garfield, 1995) and that a decrease in its activity is coincident with the beginning of the uterine contractions that are necessary for labour (Farina et al., 2001).

1.6 Preterm birth and labour

Labour is the physiologic process by which a foetus is expelled from the uterus. The process involves regular, painful uterine contractions which increase in intensity, duration and become more frequent leading to cervical dilation (Kota et al., 2013). Normal labour occurs around 37 to 42 weeks of pregnancy.

Preterm birth is defined as birth before 37 weeks of pregnancy. It is the largest cause of perinatal morbidity and mortality, and the high incidence has remained unchanged (Leal et al., 2016). In 2010, 14.9 million babies were born preterm accounting for 11.1% of all births worldwide (Boyle et al., 2017). The rates of spontaneous preterm birth range from about 5% in European countries to 18% in African countries (Blencowe et al., 2012). Preterm birth is a leading cause of neonatal deaths (Rubens et al., 2014) and morbidity in children less than 5 years old (Harrison and Goldenberg, 2016). The cost of preterm birth to the public sector is substantial; accounting for more than £2.9 billion (Mangham et al., 2009). Much of the cost is attributed to neonatal intensive care, continued heath care of preterm infants and educational requirements (Howson et al., 2013).

Preterm birth can be spontaneous or medically indicated. Spontaneous preterm births account for about 70% of preterm births, with women presenting with cervical dilation or preterm premature rupture of membranes (PPROM) (Rubens et al., 2014, Boyle et al., 2017). The underlying cause of spontaneous premature labour is often unknown; however certain risk factors may contribute to preterm labour and birth. They include: drug abuse, previous late miscarriages, low maternal weight at time of conception, maternal smoking, uterine abnormalities, cervical incompetence, antepartum haemorrhages, multiple gestations, previous preterm labour and infection (Vause and Johnston, 2000). The medically indicated preterm births are often precipitated by clinical conditions such as preeclampsia, diabetes, maternal medical conditions, and foetal problems (Moutquin, 2003) and are not the focus of my research since they are not normally labouring. In the western world, about one-third of infants born preterm are delivered by caesarean section or

induction of labour because of pregnancy complications, and these deliveries contribute to the growing incidence of late PTB (Rubens et al., 2014).

Several mechanisms could lead to preterm labour including inflammation, infection, ischaemia, haemorrhage or stress (Goldenberg et al., 2008). Increased maternal risk of preterm birth is associated with extremes in maternal age, multiple pregnancies, history of previous preterm births, assisted reproductive technology, family history, substance abuse, cigarette use, low socioeconomic status, low maternal pre-pregnancy weight, bacterial vaginosis, periodontal disease, and poor pregnancy weight gain (Institute of Medicine Committee on Understanding Premature and Assuring Healthy, 2007). The biological basis for some of these risk factors is poorly understood (Rubens et al., 2014). Although many factors including sociodemographic, nutritional, biological, and environmental can increase the risk of spontaneous preterm birth, the cause is not fully understood (Muglia and Katz, 2010, Goldenberg et al., 2008).

Preterm birth is associated with a number of health complications (Rubens et al., 2014) such as cognitive and neurological impairment such as cerebral palsy, respiratory and gastrointestinal complications (Marlow et al., 2005). There is also the increased risk of chronic diseases in adulthood, such as obesity, diabetes and hypertension (Rubens et al., 2014).

Clinically preterm labour is diagnosed by confirmation of regular uterine contractions and evidence of cervical dilation. Contractions are then managed with tocolytics thereby providing time for administration of corticosteroids to reduce preterm morbidity and mortality. Despite the high burden of preterm birth on childhood mortality, morbidity, and health care expenditure, few strategies are in place to effectively prevent preterm delivery.

1.7 Treatment of Preterm labour

Tocolysis, which is the inhibition of myometrial contractions, is currently the primary treatment for preterm labour and/or birth. Since uterine contractions are one of the most recognised signs of preterm labour, researchers have focused on development of tocolytics to inhibit uterine contractions, thereby preventing preterm birth. Quantitative studies have demonstrated that all tocolytic drugs are able to delay delivery for 48 hours and 7 days, although not at delaying delivery until 37 weeks (Haas et al., 2009). The delay is sufficient for antenatal corticosteroids to be administered or for the mother to be transferred to tertiary care facility (Haas et al., 2009, Haas et al., 2012). Unfortunately, no single tocolytics has been able to effectively and optimally inhibit myometrial contractions without causing maternal and foetal side effects (Gyetvai et al., 1999). The 2012 World Health Organisation report *Born Too Soon* highlighted the need for effective tocolytics (Howson et al., 2013).

A range of tocolytics has been used to delay labour or treat preterm labour including: calcium channel antagonists e.g. nifedipine; magnesium sulphate; cyclooxygenase inhibitors e.g. indomethacin and nimesulide; β-adrenergic receptor agonists e.g. terbutaline and ritodrine; oxytocin receptor antagonists e.g. atosiban and barusiban; and others. Each tocolytic a unique mechanism of action (Figure 1.6), side effects and degree of complexity of administration (Goldenberg, 2002).

1.7.1 Calcium channel antagonists

Calcium channel antagonists act on the L-type calcium channels preventing calcium influx and the spread of action potential required for the myometrium to contract in a coordinated manner (Arrowsmith et al., 2010). This leads to a reduction in intracellular calcium, inhibiting MLCK phosphorylation, followed by decreased myosin-actin interaction promoting myometrium relaxation.

Nifedipine is a smooth muscle relaxant which reduces the intracellular influx of calcium ions. It acts by binding to the inside of the L-type VGCCs causing them to remain closed. Though not a licensed tocolytic, nifedipine is recommended by the NICE as first line tocolytic (NICE, 2015). Studies have shown that nifedipine is able to

delay labour for up to 7 days (van Geijn et al., 2005). A systematic review of 12 randomised controlled trial involving 1029 women (King et al., 2003) revealed that in comparison to other tocolytics, calcium channel blockers reduced the number of deliveries within 7 days and prior to 34 weeks gestation.

Although nifedipine is able to delay labour and is more cost effective than other tocolytics, its cardiovascular side effects remain a major concern and therefore not recommended for patients with cardiovascular morbidity (Jaju and Dhabadi, 2011).

1.7.2 Magnesium sulphate

Magnesium, a bivalent cation is the fourth most common cation in the human body (Mercer and Merlino, 2009). Although the effect of $MgSO_4$ on the duration of uterine contraction was first described about six decades ago (Hall D, 1959), the exact mechanism of magnesium as a tocolytic is not fully understood. Magnesium is thought to inhibit contractility by competing with Ca (Arrowsmith et al., 2010). Magnesium hyperpolarises the plasma membrane and inhibits myosin light-chain kinase activity by competing with intracellular calcium, which in turn reduces myometrial contractility (Simhan and Caritis, 2007). Magnesium is thought to act via both intracellular and extracellular mechanisms, leading by blocking L-type calcium channel entry and blocking agonist-stimulated IP_3 channels (Mercer and Merlino, 2009).

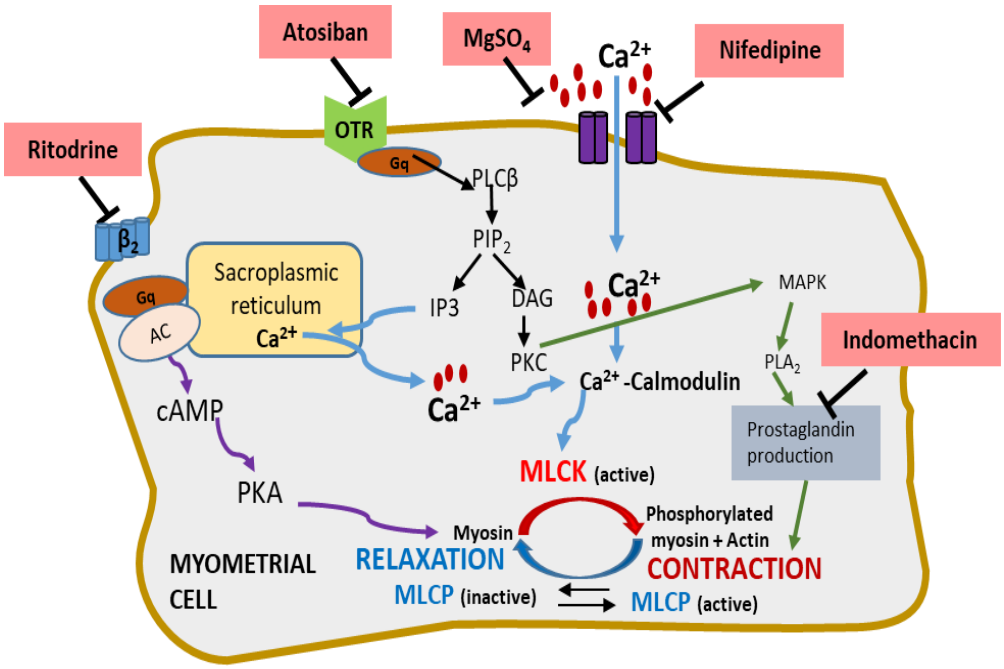


Figure 1.6: The mechanism of tocolytics on myometrial contractions Oxytocin binds GPCR leading to the activation of G-protein, and the cascade of events lead to contractions. Atosiban, an oxytocin receptor antagonist competes with oxytocin for its receptors. Indomethacin inhibits the enzyme responsible for converting arachidonic acid to prostaglandin –cyclooxygenase. Indomethacin may also act by blocking calcium channels. Nifedipine and Magnesium are both classed calcium channel blockers, blocking L-type calcium channels and calcium entry. Nifedipine prevents the reuptake of calcium ions through. Magnesium sulphate ($MgSO_4$) inhibits myometrial contractions by competing with calcium ions for the calcium channel. Ritodrine, a β-agonist, acts by increasing cyclic adenosine monophosphate (cAMP), leading to relaxation.

1.7.3 Cyclooxygenase inhibition

Cyclooxygenase (COX) inhibitor, also known as prostaglandin synthase inhibitors are drugs that inhibit uterine contractions by preventing PG synthesis, e.g. indomethacin. Prostaglandins have been proposed to play an important role in the onset and maintenance of labour, acting as myometrial stimulant (Olson and Ammann, 2007). Both term and preterm labour are associated with increased prostaglandin synthesis within the uterus (Skinner and Challis, 1985). COX enzymes are fundamental to the production of prostaglandins and inhibitors reduce uterine contractions (Doret et al., 2002). There are two COX isoforms, COX-1 and COX-2 which convert arachidonic acid to PGH_2. PGH_2 serves as a substrate for the production to the alternative prostaglandins and prostacyclins.

In vitro data have demonstrated the inhibition of uterine contractions with COX inhibitors (indomethacin and nimesulide), but not inhibition of PG; however, they reduced the Ca^{2+} channel current in human myometrium (Sawdy et al., 1998). This study also concluded that, as there was no relationship between myometrial inhibition and PG production.

1.7.4 β-adrenergic receptor agonists

The β-adrenergic receptor (β-AR) agonist inhibits myometrial contractions by binding to β-AR. Once β-AR is bound, it in turn activates adenylate cyclase by stimulating a G protein. The adenylate cyclase enzyme converts ATP to cAMP, leading to the increase of intracellular cAMP levels, which in turn activates protein kinase A (PKA). This ultimately leads to the inactivation of myosin-light chain kinase, thereby reducing contractions.

With the activation of PKA, there is decreased influx and increased efflux of calcium ions, and since $[Ca^{2+}]_i$ is the major regulator of the actin-myosin cross bridge, the effect of the reduce $[Ca^{2+}]_i$ levels is myometrial relaxation. Thus, through activation of protein kinase A, the beta agonist causes muscle relaxation in two ways: inhibition of myosin light chain phosphorylation and decreasing $[Ca^{2+}]_i$ levels (B. Caughey and T. Parer, 2001).

Of the sub-types β-AR, β_2-AR is the most predominant making up to about 80%; moreover, this changes depending on the expression at different gestations and physiological differences. It has been postulated that a down regulation of β_2-AR may play a role in the onset of preterm labour, although the mechanism is unclear (Arrowsmith et al., 2010).

1.7.5 Oxytocin receptor antagonists

As the name implies, oxytocin receptor antagonists are drugs that act as antagonists to OTR. They block the activation of the receptors, preventing the stimulatory effect of oxytocin, leading to myometrial relaxation. Following activation, Gαq stimulates PLC, which then generates DAG and IP3 through the cleavage of PIP2. IP3 is then released and binds to calcium channels located on the SR; this results in increased $[Ca^{2+}]_i$ levels and ultimately smooth muscle contraction (Gimpl and Fahrenholz, 2001).

Atosiban is a synthetic analogue of oxytocin and therefore a competitive antagonist. It competes with oxytocin for binding to the receptors in myometrium and decidua and prevents the increase in $[Ca^{2+}]_i$. A Cochrane review showed that atosiban is effective at delaying birth but did not improve the incidence of preterm births before 37 weeks nor improve neonatal outcome (Papatsonis et al., 2005).

1.8 Dual Tocolysis

Although studies show that tocolytics prolong pregnancy in threatened preterm labour, the use of tocolytics is not without severe concentration-dependent side effects (Berkman et al., 2003). Drugs like calcium channel blockers or prostaglandin synthesis inhibitors have high efficacy with low cost, but result in many side effect (Maitra et al., 2007, Olson and Ammann, 2007). On the other hand, oxytocin receptor antagonist, even though are safe and effective agents, are also very expensive (Wex et al., 2009, Al-Omari et al., 2006). As shown in Figure 1.3, each tocolytic acts via a unique intracellular pathway, creating opportunities to explore combining tocolytics especially those acting via different pathways.

The combination of tocolytics has been investigated in both animal and human myometrium. A recent Cochrane review (Vogel et al., 2014a) pointed that in comparison to single agents, combining tocolytics especially those acting via different mechanistic pathways may have a greater potential in increasing efficacy. Furthermore, combined tocolytics may result in reducing dosage and frequency of administration thereby reducing side effects of each agent. The authors also suggested the urgent need to explore the effect of combining tocolytics compared to single agents (Vogel et al., 2014a). In agreement with their suggestion, this work focuses on combining magnesium with most commonly used tocolytics in the UK.

1.9 Aims of the Thesis

Due to the steady rise in preterm birth, and the lack of efficacy of single tocolytics, a recent Cochrane review suggested combining tocolytics. In this thesis, I will investigate the hypothesis that combining two agents acting via different mechanism may produce a synergistic relaxant effect on myometrial contractions. Therefore, the aims of my thesis were to:

(i) investigate the effect of magnesium on spontaneous contractile activity in mouse myometrial contractility,

(ii) compare the effect of magnesium in oxytocin-induced contractions, and

(iii) investigate how the tocolytic efficacy of magnesium is altered with gestational changes,

(iv) determine if the tocolytic efficacy in different gestations is altered in the presence or absence of oxytocin;

(v) To determine the effect of indomethacin, atosiban, and nifedipine on spontaneous and oxytocin-induced contractions, and calculate their respective IC_{50} values;

(vi) To examine the effect of magnesium in combination with indomethacin compared to Mg alone;

(vii) To examine the effect of magnesium in combination with atosiban compared to magnesium alone;

(viii) To examine the effect of magnesium in combination with nifedipine on term pregnant mouse uterine contractility compared to Mg alone;

(ix) To determine which combination of tocolytic is most effective in reducing term pregnant mouse uterine contractions;

(x) determine if L-type calcium subunits (Cav 1.1 and 1.2) are expressed across different gestations of the mouse uterus; and if present,

(xi) quantify the mRNA expression of L-type calcium subunit at different gestational states using real time qPCR;

(xii) examine the most commonly used reference genes in mouse uterus; and

(xiii) identify the most stable housekeeping genes for qPCR across mouse gestational states.

Chapter 2

General Materials

And Methods

Chapter 2

General Materials and Method

This chapter will provide a general description of the apparatus and methods used in this thesis. A more detailed explanation of the methods and chemicals used will be given in the relevant chapters.

2.1 Animals and Tissue Preparation

2.1.1 Animal Housing

Only female CBL57/6J mice (Charles River, UK), were used in the work performed in this thesis. Pregnant mice were individually housed in open cage while non-pregnant mice were kept in a group of four. The animals were on a 12-hour light/dark cycles (7am to 7pm), with access to standard laboratory food; CRM pelleted (SDS Ltd, UK), and animals were provided with water *ad libitum*. Room temperature was kept at 21 ^{o}C ± 2 ^{o}C, and the humidity was kept relatively between 40-55% as specified by Home Office regulations, UK.

2.1.2 Animal Ethics and Regulation

All animals were handled and killed according to the code of practice for humane killing of animals under the Schedule 1 (Scientific Procedures) Act 1986. The mice were placed in a chamber and anaesthetised with rising concentration of CO_2. Death was then confirmed by cervical dislocation. If pregnant mice were used, the pups were delivered by caesarean section and killed by cervical dislocation.

2.1.3 Uterine Tissues

In this study, mice tissues were used as they are easier to obtain at different gestations. Uterine tissues were obtained from non-pregnant (weighing 175-200g, age of approximately 8-10 weeks), 14-day pregnant, 16-day pregnant, term-

pregnant (18-19 days gestation), or postpartum day 1 mice. (Parturition in mice occurs between days 19 to 20 of gestation).

Female CBL57/6J mice (Charles River, UK) were time-mated with the male mice taken in/taken out of the cage being classed as Day 0/1. When pregnant, mice at different gestational ages were humanely killed and dissected accordingly. Term tissue was taken from mice that were within 24 hours of labour (Day 19/20). Uterine tissues were obtained at approximately the same time (usually between 8.30am- 9.30am). Non-pregnant uterine tissue was obtained from virgin CBL57/6J mice.

2.1.4 Collection, Dissection and Storage of Uterine Tissue

The uterus was removed and immediately placed in a buffered physiological saline solution (PSS).

After isolating the uterus, it was then placed on a petri dish containing PSS under a light microscope. The uterus was cut open with the endometrium side facing downwards. Any excess blood, fat, placenta or connective tissue was cleaned from the uterus. Longitudinal uterine strips measuring approximately 1mm x 2mm x 10mm were then dissected. Each strip contained endometrium, perimetrium and myometrium.

If the strips were to be used for RNA extraction, they were weighed and placed in RNAlater (Thermo Fischer Scientific, US) at 4^{o}C. After about 24 hours, the strips were taken out from the RNAlater and placed in cryo-vials. They were then stored in -80^{o}C until enough samples were collected to begin RNA extraction. If uterine strips were used in contractility, each strip was tied at both ends with surgical silk.

2.2 Chemicals and Solutions

All solutions were purchased from Sigma (Poole, Dorset UK) unless otherwise specified.

2.2.1 Physiological Saline Solution (PSS)

The PSS/Krebs was prepared freshly daily and had the following composition:

Composition	Concentration	Mass concentration
Sodium chloride (NaCl)	154mM	9.00 g/L
Potassium chloride (KCl)	5.6mM	0.42 g/L
Magnesium sulphate heptahydrate ($MgSO_4$ $7H_2O$)	0.12mM	0.29 g/L
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES)	10.9mM	2.60 g/L
Glucose	8mM	2.10 g/L
Calcium chloride ($CaCl_2$)	2.0mM	2ml/L
Distilled H_20 (dH_20)		1L

Table 2. 1: Components and Composition of Physiological saline Solution

Using 4M Sodium Hydroxide (NaOH), the pH of the solution is then adjusted to 7.4.

2.2.2 Acidic PSS

This was made using the same composition as PSS, however the pH was adjusted using 1M Hydrochloric acid (HCl). The buffer was not changed as HEPES buffer is effective between pH 6.8 and 8.2. The Acidic pH needed in this work was 6.9.

2.2.3 PSS with Increased Concentrations of $MgSO_4$

For magnesium and combined tocolytics experiments, increasing concentrations of $MgSO_4$ were added to PSS. Table 2.2 shows the concentration of $MgSO_4$. Cumulative doses (2-10 mM) of $MgSO_4$ were added to PSS on spontaneous contractions while 2-12 mM of $MgSO_4$ was applied on oxytocin-induced contractions. Details of magnesium's application for the different protocols are explained within the relevant result chapters 3 and 4.

2.3 Drugs and Agents

Preparation of drugs used in the contractility experiments and the methods of application to the organ bath are described in this section. Application of all drugs was done after a steady state of contraction was reached.

Concentration	Mass concentration (g/100ml)
2mM	0.0196
4mM	0.0686
6mM	0.1176
8mM	0.1666
10mM	0.2156
12mM	0.2646

Table 2. 2: Concentration of MgSO4 added to PSS

2.3.1 Oxytocin

Oxytocin was dissolved in dH_20 to make a stock of 10mM concentration. It was then aliquoted in small vials and stored at -20^oC, defrosted and diluted in PSS as needed on day of experiment. The PSS was then diluted to the desired concentrations. For non-pregnant myometrium, a concentration of 1.0nM was used while 0.5nM was used for pregnant uterine tissues. A lower concentration of oxytocin was chosen in pregnant mouse myometrium, as previous work in mouse showed that less was required to produce optimal stimulation (unpublished data). A 1.0nM concentration in pregnant myometrium would produce tonic contractions in most strips.

2.3.2 Atosiban

Atosiban was dissolved in Bovine Serum Albumin (BSA) to produce 1mM stock solution, as per manufacturer's protocol. It was then aliquoted into small vials and stored at -20^{o}C until used. 1mM working concentration was used for experiments by diluting the stock solution with Krebs and oxytocin. At the beginning, I was trying to find the best physiological concentration of atosiban that could block the OTRs but not affecting the spontaneous uterine activity. A concentration response was carried out between 10nM -3µM and the IC_{50} value was determined. Consequently, I decided to use 500nM final working concentration of Atosiban in my experiments to block the OTRs but not affecting the overall uterine activity.

2.3.3 Indomethacin

Indomethacin was dissolved in dimethyl sulfoxide (DMSO) to produce 100mM stock solution and then stored at room temperature according to manufacturer's guides. A concentration response was carried out from 1 µM -100 µM and IC_{50} was determined for both spontaneous and oxytocin-induced contractions. 3 µM and 30 µM working concentration for spontaneous and oxytocin-induced contractions respectively was used for experimentation by diluting the stock with Krebs' solution. This concentration was chosen carefully, so that should have the maximum effect on cyclooxygenase without affecting the overall uterine activity.

2.3.4 Nifedipine

Nifedipine (Sigma, UK) was dissolved in ethanol to produce a 1mM stock solution. The stock solution was stored at 4^{o}C until required. A concentration response test was carried out from 0.1 nM – 30 nM to determine the IC_{50} values.

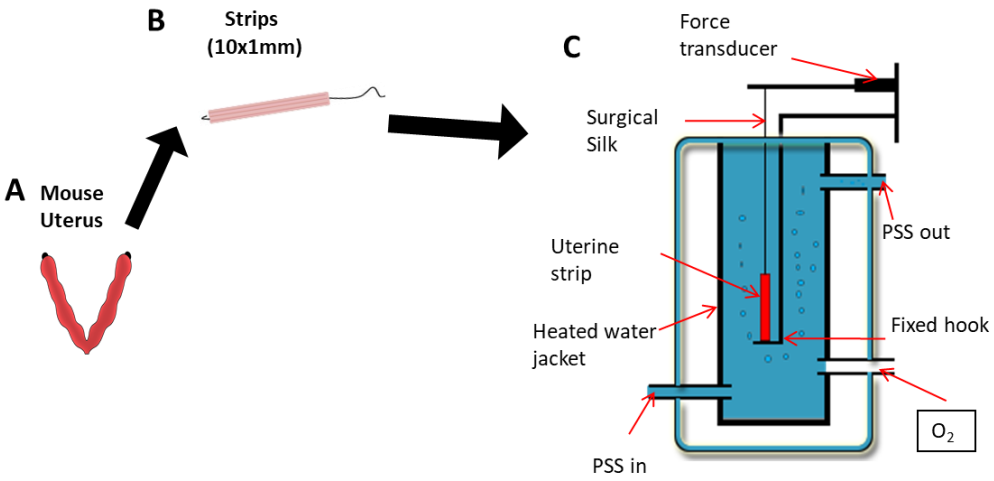


Figure 2. 1: Recording of contractile activity using an organ bath

Uterine strips, measuring 1mm x 2mm x 10mm in size, are dissected from mouse (**A**). Surgical strings are tied to the strips (**B**) with one end attached to a fixed hook and the other to a force transducer of an organ bath (**C**). This was superfused with physiological saline solution at 37^{o} C, pH 7.4. Contractility was then measured.

2.4 Isometric Tension Measurements

Firstly, force was calibrated in Newtons (N) using a weight (1g). The 1g weight was suspended on the hook connected to the force transducer and the deflection was measured. A 1g weight is 9.81 mN (N is kg/ms^2).

Tissues were tied with surgical silk with one end attached to a fixed hook and the other to the force transducer in 5ml organ bath (Linton Instruments, UK) containing PSS (Figure 2.1). The tissue strips were then stretched by 5mN. Strips were continually superfused with PSS solution, and the temperature was adjusted and maintained at 37^oC throughout the entire experiment using Thermostatic bath (Grant Instruments Ltd, Cambridge). The force transducer (World Precision Instruments (WPI), UK) was attached to a Digidata acquisition package and Datatrax software (WPI instruments, UK). These strips were left to equilibrate for at least 45 minutes before any experiments began. I established that mouse myometrial tissue could sustain contractions for many hours (Figure 2.2).

During periods of control and application of drugs, four contractile parameters were analysed. These parameters were force amplitude, frequency, duration and area under the curve (AUC). Figure 2.3 shows how these parameters were measured. The analysis of these parameters was done using the Origin Pro Software (Origin version 2016). These parameters were normalised against the control (100%) values.

Force amplitude

The force amplitude of contractions was determined by measuring the baseline value of each contraction and subtracting it from the value of the peak of that contraction. The force amplitude is recorded in mN. If there were no contractions, the force was taken as '0'.

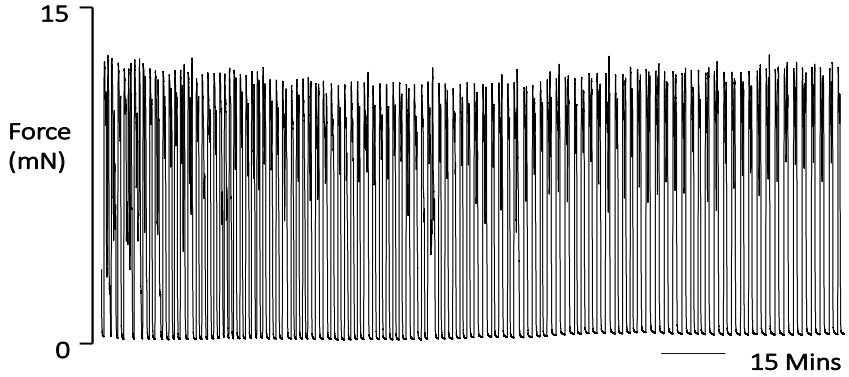


Figure 2. 2: Control contractility trace

Representative isometric trace showing over 2 hours of spontaneous contractions from mouse myometrium at 37^{o}C with perfusion rate 4ml/min

Frequency

The frequency of contractions is a measurement of how many contractions happen over a given period. The frequency of contractions is recorded as number of contractions/ x minutes, where x was often 10 minutes. If there were no contractions, the frequency was taken as '0'.

Duration

The duration is the amount of time each contraction lasted for. This was measured by recording the time at half-maximal height of contractions (50% of force amplitude). The duration of contractions was recorded in minutes. If there were no contractions, the duration was not measured.

Area under the curve (AUC)

The AUC is also referred to as mean force integral. The AUC takes into account all the other contractile parameter together. This parameter was measured using the Origin Pro 2015 software (Origin Lab Corporation, MA, USA) . If there were no contractions, it was recorded as '0'**.**

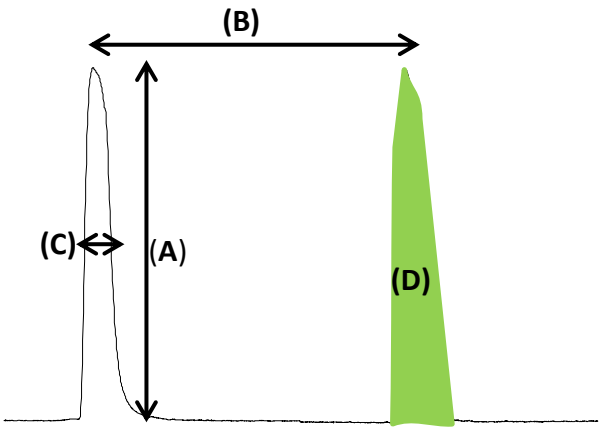


Figure 2. 3: Illustration of the measurement of contractile parameters

(A) Amplitude measured from baseline to peak of contraction (mN); (B) frequency measured by the time difference between the peak of contractions (number of contractions/minutes); (C) duration measured by time taken from the beginning of a contraction till it relaxes; and (D) force integral determined by the area under the curve of a contraction.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 RNA extraction

Preparation of RNA was achieved using TRIzol reagent (Invitrogen TRIzol® Plus RNA Purification kit), a solution that contains phenol and guanidine isothiocyanate, which helps to maintain the integrity of the RNA, while allowing for complete dissociation of nucleoprotein complexes. This procedure was carried out as per manufacturer's protocol with a few changes.

TRIzol (1mL) was added directly to uterine samples (~100mg) and homogenization was carried out using a tissue homogenizer. The sample lysate was incubated at room temperature for 5 minutes to allow complete dissociation to occur, then 0.2mL chloroform was added, mixed vigorously by hand for 15 seconds and incubated for a further 2-3 minutes at room temperature. After centrifugation (12,000g) for 15 minutes at 4° C, there was a 3-layer separation consisting of a lower, red phenol-chloroform phase, a middle phase and the colourless aqueous upper phase (containing the RNA) which was transferred to a fresh tube.

RNA was precipitated from the supernatant by addition of equal amount of 70% ethanol and vortexing. Following centrifugation at 12,000g for 15 seconds at room temperature, the supernatant was then removed and discarded. This step was repeated until the entire sample was processed. 0.7mL of wash buffer was added and centrifuged at 12,000g for 15 seconds, while discarding the supernatants. 0.5mL of wash buffer II (mixed with ethanol) was also added, and centrifuged at 12,000g for 15 seconds at room temperature, discarding the supernatant.

To dry the membrane, the spin cartridge and collection tube (provided in the kit) were centrifuged at 12,000g for 1 minute at room temperature. This collection tube is then discarded and spin cartridge was then placed in a recovery tube. 30µL of RNase free water was added to the spin cartridge and allowed to incubate at room temperature for 1 minute. This is followed by centrifugation at no less than 12,000g for 2 minutes at room temperature. The spin cartridge was discarded while the flow

through was placed immediately on ice if used within a few hours or at -80^{o}C for long term storage.

2.5.2 RNA Quantification

Following the isolation of RNA, quantification of RNA was carried out using a nanodrop ND-1000 spectrophotometer (Thermo Fischer). This step was done to determine the purity and integrity of the RNA samples.

2.5.3 DNase Treatment

This procedure was carried out using the TURBO DNA-*free* Kit (Ambion by life technologies). This kit included all reagents used (TURBO DNase, 10X TURBO DNase buffer, DNase inactivation reagent and nuclease free water). DNase treatment aids the removal of genomic DNA contamination from the RNA preparation before reverse transcription. It also helps removal of divalent cations that could increase RNA degradation when heated. According to the manufacturer's protocol, a reaction size of between 10 - 100µL was recommended, therefore in this thesis, a 50µL reaction size was used.

Using the concentration of RNA samples gotten from the nanodrop data, the volume of RNA for 10µg in 50µL reaction was calculated. 10x TURBO DNase buffer (5µL) and TURBO DNase (1µL) were then added to the predetermined volume of RNA and mixed gently. The remaining volume was made up with RNase free water. Following incubation of the mixture at 37^{o} C for 20-30 minutes, 5mL of the resuspended inactivation reagent was added and mixed. This was then incubated for 5 minutes at room temperature while flicking occasionally. The mixture was then centrifuged at 10,000g for 1.5 minutes and the supernatant (containing the RNA) was carefully transferred into a fresh tube.

2.5.4 cDNA Synthesis or Reverse Transcription

Reverse transcription (RT-PCR) utilises the ability of the enzyme reverse transcriptase to synthesize DNA from an RNA template. The resulting complementary DNA (cDNA) fragments represent the population of genes which are expressed in the tissue from which the RNA was extracted. Since the messenger

RNA molecules will have been processed and introns excised from the sequence, cDNA can be used to study the transcripts present in a given tissue type. cDNA was prepared using the AMV First Strand cDNA Synthesis Kit (BioLabs Inc.). The kit was stored at -20^0 C and experiment was carried out as per manufacturer's protocol. The AMV kit composed of AMV enzyme mix, AMV reaction mix, Oligo d(T)$_{23}$ VN, random primer mix and nuclease free water.

A total of 5ul of RNA was added to 2 µL of Random primer mix (60 µM). The reaction volume was made up to 8µL with nuclease free water. To another tube, 10 µL of AMV reaction mix was added to 2 µL of AMV enzyme mix. The 8 µL of ample mix was then added to this tube to make a total of 20 µL reaction. To a separate tube (negative control), 10 µL of AMV reaction mx was added to 2 µL of nuclease free water. Again, this was added to the 8 µL of sample mix.

The 20 µL cDNA synthesis reaction was then incubated at 25^o C for five minutes before being incubated further at 42^0 C for one hour. Following this, the reaction was terminated by inactivating the enzyme at 80^0 C for five minutes. The reaction volume was made up to 50 µL by the addition of 30 µL nuclease free water and stored at -20^o C.

2.5.5 Primers

The sequence of calcium channel subunit primers (Sigma Aldrich Co.) used in the conventional PCR stage was obtained from the literature (Xu et al., 2003). Each primer was initially at 100µM of forward and reverse (separately). A combination of forward and reverse primers was done, reconstituting primers to 5µM working stock. When used in PCR reaction, a concentration of 0.5 µM was used.

For establishing the optimal housekeeping genes, a commercial kit geNorm reference gene selection kit was purchased from Primer design UK. The kit contains 12 genes which have been determined from over 13000 microarray experiments to be stably expressed across different tissues, experimental conditions, and different disease condition. The kit includes classically used housekeeping genes for comparison.

Primers for Cav 1.2 were also designed by Primer Design UK. The forward and reverse primers were reconstituted and 0.3 µM concentration (F+R) was used in qPCR reaction.

2.5.6 End-Point or Conventional PCR

For end-point PCR, the HotStarTaq® Master Mix kit (Qiagen®) was used. HotStarTaq® Master Mix is supplied at 2x final concentration, with the final reaction concentrations as follows: 2.5 units HotStarTaq DNA polymerase, 1x PCR buffer (contains 1.5 mM $MgCl_2$) and 200 µM of each dNTP. The forward and reverse primers were added to the master mix at a final concentration of 0.2µM of each primer and template DNA at < 1 µg/ 50 µL reaction. The exact concentrations of primer and templates were adjusted to optimise the reaction. The remainder of the reaction volume was made up with nuclease free water. An example of the volumes used a typical reaction using HotStarTaq® Master Mix is shown in Table 2.3. For my experiments, a 10 µL reaction mix was made.

Components	Volume used for 10 µL reaction	Final concentration
HotStarTaq master mix	5µL	2x
Primer mix: includes forward primer (10µM) + reverse primer (10 µM)	1 µL (F+R)	5 µM
Template DNA	1 µL	200ng/10 µL reaction
RNase free water	3 µL	-

Table 2. 3: Preparation of a 10µL PCR reaction using HotStarTaq® Master Mix

The PCR tubes containing the 10 µL reaction were placed in Bio-Rad T100 Thermal cycler (Bio-Rad Laboratories Inc. USA) and PCR conditions were adjusted to optimise

the reactions. Table 2.4 shows the optimized cycling conditions used in my experiments.

	Step	Time	Temperature
1.	Initial heat inactivation	15 minutes	95° C
2.	Denaturation	1 minute	95° C
3.	Annealing	1 minute	50-70° C
4.	Extension	2 minutes	72° C
5.	Number of cycles	Repeat steps 2 to 4 (34 times)	-
6.	Final extension	10 minutes	72° C

Table 2. 4: PCR conditions for DNA amplification

The thermocycler ends the amplification process by entering a state of indefinite cooling at 4°C. Samples were stored at 2 - 8° C for use the following day.

2.5.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used in my research to determine the presence and size of PCR products. 1% agarose (Sigma) was made by adding 1 gram of agarose powder to added to 100ml of 1X TAE buffer (consisting of 4.84g Tris base, 1.14ml glacial acetic acid, 2ml 0.5M EDTA, pH 8). Using a microwave, the mixture was allowed to heat and melt on medium power and 10 µL SYBR™ safe DNA gel stain (Thermo Fischer Scientific) was added to allow visualisation of DNA fragments under long-wave ultraviolet light. The melted agarose solution is the poured in gel casting tray and the appropriate combs inserted. After the gel was cooled and become solid, the combs were then carefully removed and the gel trays placed in the electrophoresis chamber. The 1x TAE buffer was poured into chamber so that there is about 2-3mm of buffer over the gel.

In loading the gel, 2µL of 6x sample loading buffer (BioLabs Inc.) was added to each 10 µL PCR reaction. 5 µL of sample was pipetted in separate wells. To provide a reference for size, 1.5 µL of DNA ladder standard was pipetted in at last one well on each row on the gel. For this thesis, 50bp and 100bp ladder were used (BioLabs Inc.).

Electrophoresis was carried out at 110 volts for about 60-90minutes depending on the size of products being resolved. Following electrophoresis gels were visualised on an ultraviolet transilluminator and images were captured using the capture feature of the software, UVP Chemi TS software (Analytik Jena, US).

2.5.8 Choice of reference or housekeeping genes

Several statistical tools, including geNorm, Normfinder and Bestkeeper are available to identify the most stably expressed genes. Studies have shown that in most cases there is no significant difference between the different tools (De Spiegelaere et al., 2015, Wang et al., 2002) as studies using them have shown very similar results. The geNorm software (geNorm package v 3.5) was selected and used in this thesis to select the most stable genes.

GeNorm is an algorithm that determines the most stable reference genes from a set of tested candidate genes in a given sample panel. A gene expression normalisation factor can be calculated for each sample based on the geometric mean of a user defined number of reference or housekeeping genes.

Pairwise variation (Vn/n+1) of each gene is determined as standard deviation of the logarithmically transformed expression ratios. This is calculated to evaluate the minimum number of reference genes required for normalization. A pairwise variation of 0.15 is considered the cut-off value below which the inclusion of additional reference gene will not be required (Vandesompele et al., 2002). An M value is calculated for a particular gene as the arithmetic mean of pairwise variation with all other genes in the same panel. Genes that are stably expressed are characterised by the lowest M value. Stepwise exclusion of the least stable genes results in combination of two constitutively expressed reference genes (Vandesompele et al., 2002)

My qPCR results were normalised to reference or housekeeping genes, and therefore results are an expression of the relative quantification of the gene of interest in relation to the endogenous control.

2.5.9 Quantitative real-time PCR

Quantitative real-time PCR assay is a reliable and fast technique for the quantification of transcription levels in gene expression studies, and are used frequently in many areas of modern research. This technique provides quantification of input templates over a broad linear range, low sample consumption, rapid throughput of large sample numbers, and low risk of contamination (Gibson et al., 1996, Livak et al., 1995).

Using SYBR green chemistry, real-time PCR was performed with CFX Connect Real-time system (BioRad laboratories, Hercules, CA, USA). Details on the amplification mixture are specified within chapter 5.

Relative standard curves for target genes and housekeeping genes were constructed from a serial dilution of cDNA template. Both samples and standards were run in triplicates on 96-well Hard Shell® PCR thin-wall plates (Bio-Rad laboratories Inc. USA), with every run including a no template control (NTC or no cDNA). An optimized volume of cDNA was used for the amplification of each gene. Precision ® FAST qPCR Master Mix was used for qPCR and the samples were treated according to the manufacturer's instructions (Primer Design, UK). The reaction components are detailed in the result chapter.

2.5.10 Analysis and determination of target gene expression relative to housekeeping gene

Accurate normalisation is fundamental to obtaining sound results. Normalisation is usually achieved by simultaneous amplification of housekeeping genes along with the target gene.

For every sample, the cycle threshold (Ct) value was determined using the CFX Software (Bio-Rad laboratories Inc. USA). The threshold value indicates background level of fluorescence. The Ct value indicates the number of cycles at which the fluorescence passes the set threshold value and is therefore a way to determine the concentration of the gene of interest.

Since my samples were run in triplicate, the Ct values were then determined for each well and the mean Ct value was used, and where Ct values differed by more than 1.2, they were excluded and samples repeated. The expression of target gene was then determined relative to the housekeeping gene value for each sample. Generally, the lower the Ct values, the greater the amount of gene expression.

2.6 Sample Size in this Thesis

The sample size is the number of uterine strips used from each mouse. In this thesis, there is variation in the samples size between different protocols. We considered using power calculation; however determination of n numbers was taken from previous animal studies by us and others. Power calculations are more generally used in clinical studies, where there can be very large sample sizes and small, but important, effects. In biomedical science experiments, previous knowledge of statistical variation in control parameters, e.g. amplitude of contraction, and estimates of effect size, guide the choice of n numbers. Generally, the focus was to do the experiments by using as large a number as needed for significance and to uphold the guidelines set by the UK home office legislation for animal use. As a result, it is important to kill the smallest number of animals consistent with obtaining meaningful data The sample size was calculated retrospectively using the formula: $n= 2(Z^2\ \sigma^2/E^2)$ where **Z** is the value from the standard normal distribution reflecting the confidence level that will be used (e.g., Z = 1.96 for 95%), **σ** is the standard deviation of the outcome variable and **E** is the desired margin of error. The formula used generates the minimum number of samples required to ensure that the margin of error in the confidence interval does not exceed E. Therefore with an error of 0.05 (5%) with a power of 0.5, an alpha significance level of 0.05 with a 95% Confidence interval, power analysis indicated similar sample size as that used: at least an *n* of 4 in pH and nifedipine protocols and the others at least an *n* of 8.

2.7 Data and Statistical Analysis

Cumulative concentration-response curves were fitted to the logistic equation using non-linear regression. The 50% inhibition concentration (IC_{50}) was calculated for each tocolytic using the sigmoidal dose–response equation "log [inhibitor] vs. response – variable slope". The –log IC_{50} values between groups were compared on the basis of the extra sum of squares F-test. The top plateau of the curve was constrained, and the effects of the drugs on Log IC_{50} values and steepness of the curve (Hill slope) for AUC activity were analysed by GraphPad Prism 6.0 (GraphPad

Inc) using a sigmoidal dose response (variable slope) to test the null hypothesis that log IC_{50} and Hill slope are the same for each data set. Statistical significance between the values was calculated using ANOVA and the appropriate post hoc test for multiple comparisons was chosen (Bonferroni comparison for multiple pairs) at each individual concentration of the tocolytics (antagonist) used.

In general, graphs and tables were constructed using Microsoft Excel 2016, Origin Pro 2016, and GraphPad Prism 6.0. Data were represented as Mean ± standard error mean (SEM). For all tests, significance was taken as a p value less than 0.05 ($p<0.05^*$).The specifics of statistics for each chapter are discussed within the results chapter.

Chapter 3

Gestational and Hormonal Effects on Magnesium Sulphate's Ability to Inhibit Mouse Uterine Contractility

Chapter 3

Gestational and Hormonal effects on Magnesium Sulphate's Ability to Inhibit Mouse Uterine Contractility.

3.1 Abstract

Magnesium sulphate is used as a tocolytic, but clinical efficacy has been seriously questioned (Grimes and Nanda, 2006). The main objective was to use controlled *ex vivo* conditions and known pregnancy stages, to investigate how two key factors, hormones and gestation, affect Magnesium's tocolytic ability. It was hypothesised that these factors could underlie the varying clinical findings around Magnesium's efficacy. Myometrial strips were obtained from non-pregnant (n=10), mid-term (n=12) and term-pregnant (n=11) mouse uterus. The strips were mounted in organ baths superfused with oxygenated physiological saline at pH 7.4 and 37 °C. The effect of different concentrations of $MgSO_4$ (2 – 20mM) was examined on spontaneous and oxytocin-induced (0.5-1nM) contractions. Contractile properties (amplitude, frequency and area under the curve, AUC) were measured before and after application of Magnesium. Magnesium sulphate had a concentration-dependent inhibitory effect on both spontaneous and oxytocin-induced contractions but was less effective in the presence of oxytocin. In spontaneous contractions, Magnesium was more potent as gestation progressed ($P<0.0001$). In the presence of oxytocin however, there were no significant gestational differences in its effects on contraction. The rapid onset and reversal of Magnesium's effects suggest an extracellular action on Calcium entry. Taken together I can conclude that Magnesium's actions are influenced by both gestational state and hormones, such that, at least in mice, it is least effective in early gestation with oxytocin present and most effective at term in the absence of oxytocin. That Magnesium is least effective preterm and oxytocin decreases its effectiveness throughout gestation, may explain its disappointing clinical effects as a tocolytic.

3.2 Introduction

The high incidence of preterm birth, >10% of births worldwide, remains unchanged despite much research effort and advancement in understanding uterine physiology (Cypher, 2012). It is the single largest cause of mortality and morbidity in newborns. Several tocolytics including oxytocin-receptor antagonists e.g. atosiban, prostaglandin synthesis inhibitors e.g. indomethacin, calcium channel blockers e.g. nifedipine, beta-2 agonists e.g. ritodrine, and magnesium sulphate ($MgSO_4$) have been developed to help prolong pregnancy, by reducing or slowing uterine contractions when preterm labour threatens. Unfortunately, it remains the case that none are ideal in terms of either efficacy or side effects, and perhaps not surprisingly there is little international consensus on which tocolytic to use to help manage spontaneous preterm labour.

Magnesium sulphate was described as a tocolytic in 1959 (Hall D, 1959) and shortly after became the preferred drug in treating preterm labour. More recently however, studies including Cochrane systemic reviews (Crowther et al., 2014) (Crowther et al., 2002) have suggested that $MgSO_4$ is ineffective in the treatment of preterm labour. For example Crowther et al., (2014) concluded that it was no better than placebo for the primary outcome of giving birth within 48 hours of trial entry, and nor was there any significant difference for the primary outcome of serious infant morbidity (Crowther et al., 2014). In contrast, $MgSO_4$ has been shown to be the drug of choice in treatment of seizures in eclampsia (Lucas et al., 1995) and prevention of preeclampsia in hypertensive pregnant women (Duley et al., 2010). Additionally, $MgSO_4$ has been shown to have neuroprotective effects in preterm foetuses at delivery, reducing the risk of death, cerebral palsy, and gross motor dysfunction (Doyle et al., 2009).

In contrast to clinical findings, *in vitro* studies have consistently found that Magnesium has a relaxant effect on smooth muscles including airway (Gourgoulianis et al., 2001) and vascular (D'Angelo et al., 1992). Studies in myometrial smooth muscle show it to be a relaxant in several species (Kantas et al., 2002, Kawarabayashi et al., 1989, Tica et al., 2007, Popper et al., 1989); it

significantly inhibits contractions in a concentration-dependent manner(Tica et al., 2007, Arrowsmith et al., 2016).

In smooth muscles, Magnesium inhibits contractility via multiple mechanisms including effects on extracellular calcium entry, intracellular calcium release and calcium oscillations (Phillippe, 1998). It primarily acts by competing with Ca^{2+} at the L-type, voltage-gated Calcium channel (VGCC), resulting in a decrease in intracellular calcium concentration (Phillippe, 1998) The channel is comprised of four subunits, of which α-1 is the pore forming, voltage sensitive and conducting component, and the others modulate its activity. Progression of gestation has been linked to an increase in α-1 expression, and VGCC activity increases close to term (Tezuka et al., 1995, Mershon et al., 1994, Collins et al., 2000). These findings suggest that Calcium channel density and expression increases with gestation. As Magnesium's main action is at the calcium channel, the question arises: could these changes in Calcium channel density and expression alter the effectiveness of Magnesium at different gestational ages?

Of the hormonal changes around labour, oxytocin has a pivotal role (Arrowsmith and Wray, 2014). Oxytocin stimulates myometrial activity via a variety of mechanisms, including increasing Ca^{2+} entry into the myometrium via L-type channels and stimulating Calcium release from the sarcoplasmic reticulum (SR)(Luckas et al., 1999, Matthew et al., 2004b). Oxytocin stimulation would therefore be expected to mitigate the actions of Magnesium. Thus if women with threatened preterm labour had differing levels of oxytocin or differences in levels of expression of its receptor (Blanks et al., 2007), this may also alter responsiveness to Magnesium tocolysis *in vivo*.

It would be of benefit if the *in vivo* and *in vitro* findings concerning Magnesium's effects on uterine contractility could be reconciled. This could then enable a stratification of which threatened spontaneous preterm labours may benefit from its use as a tocolytic. The approach was to consider if there may be physiological factors which affect Magnesium's efficacy *in vivo*. Preterm births are defined as those before 37 completed weeks of gestation, and thus cover a very large gestational age range and much change in myometrial physiology and hormonal

conditions(Byrns, 2014, Shynlova et al., 2009, Breuiller-Fouche et al., 2007, Garfield et al., 1998). These changes can be anticipated to also affect Magnesium‘s tocolytic ability.

While the effect of Magnesium in animal and human term-pregnant myometrium on spontaneous (Tica et al., 2007, Popper et al., 1989, Arrowsmith et al., 2016) and oxytocin induced contractions (Tica et al., 2007, Phillippe, 1998, Arrowsmith et al., 2016), has received some attention, the effect of Magnesium at different gestational stages has not been systematically investigated. In addition, neither has the influence of oxytocin on Magnesium’s action been studied throughout gestation. Furthermore, much can be learnt from mouse animal models, but only one study was found where they have been used to look at Magnesium’s relaxant effect, and that was on day 14 of pregnancy in lipopolysaccharide (LPS)-treated animals (Sugawara et al., 2007).

The aims of this study therefore were to:

(i) investigate the effect of magnesium on spontaneous contractile activity in mouse myometrial contractility,
(ii) compare the effect of magnesium in oxytocin-induced contractions,
(iii) investigate how the tocolytic efficacy of magnesium is altered with gestational changes, and
(iv) determine if the tocolytic efficacy in different gestations is altered in the presence or absence of oxytocin;

3.3 Methods

3.3.1 Solutions

All study solutions were prepared fresh at the start of each experiment by direct dissolving of $MgSO_4$ or $MgCl_2$ in buffered physiological saline solution (PSS) composed of (mM): NaCl 154, KCl 5.6, $MgSO_4$ 1.2, $CaCl_2$ 2, Glucose 8 and HEPES 10.2 (Jones et al., 2004). Basal or control $MgSO_4$ therefore was 1.2mM. Oxytocin was prepared in distilled water and added to the PSS to give a final concentration of 0.5nM (pregnant tissues) or 1nM (non-pregnant tissues). A 1nM concentration of oxytocin was used for non-pregnant tissues to ensure a similar stimulation to that seen in pregnant tissues was achieved. The concentration of water did not exceed 0.01%. All chemicals were obtained from Sigma UK.

3.3.2 Tissue collection and preparation

Uterine tissues were collected and prepared as previously described by our group (Arrowsmith and Wray, 2014). Non-pregnant, 14- and 16- day (referred to as mid-pregnant) and 18 day (term) pregnant mice were humanely killed using CO_2 anaesthesia and cervical dislocation, in accordance with UK Home Office regulations. All mice used were between 8-10 weeks old. The uterus was removed, cleaned of placentas and membranes (where applicable) and full-thickness (endometrium intact) myometrial strips (1 x 2 x 10 mm) were dissected along the longitudinal axis (Matthew et al., 2004b). Using surgical thread, individual strips were mounted between a fixed support and a 10g isometric force transducer (World Precision Instruments, UK) within a 5ml tissue bath (Linton Instruments, UK) under a resting tension of 5mN and were continuously superfused with oxygenated (95% O_2, 5% CO_2) PSS at pH 7.4, at a rate of 5mL/min and maintained at 37^oC to mimic physiological conditions(Babiychuk et al., 2004).

3.3.3 Experimental protocol

For spontaneous contractions, strips were allowed to equilibrate for 45-60 minutes until regular, frequent and equal amplitude contractions were observed. For oxytocin-induced contractions, strips achieved regular spontaneous activity as above, ahead of addition of oxytocin which remained in the superfusate throughout. The uterine strips were then exposed to increasing concentrations of $MgSO_4$ from 2 – 10 mM (spontaneous) and 2 – 12 mM (oxytocin-induced) for 15 minutes or $MgSO_4$ or $MgCl_2$ (10mM and 20mM), either in the presence or absence of oxytocin.

3.3.4 Analysis and statistics

Myometrial contractions were continuously recorded via the force transducer connected to a data acquisition system equipped with Labtrax software (World Precision Instruments, UK)(Babiychuk et al., 2004). For each recording, the amplitude, frequency and force integral (area under the curve, (AUC) of contraction were measured, both during the control period (contractions occurring in the ten minutes immediately preceding the first application of Magnesium) and during the final ten minutes of each step in the concentration-response period. Data were analysed using Origin Pro 2015 (Origin Lab Corporation, MA, USA) and are presented as percentage of control to reflect the effect of Magnesium, where the control period is taken as 100%. Values given are mean ± standard error of the mean (SEM) unless stated otherwise and were compared by ANOVA, *n* is the number of myometrial tissue strips, each one from a different animal, N.
Concentration-response curves for AUC were fitted to the logistic equation with the use of non-linear regression. The concentration at which $MgSO_4$ caused a 50% reduction (IC_{50}) in overall contractile activity (AUC) was calculated. Log IC_{50} values were compared by the extra sum of squares F test or ANOVA followed by Bonferroni post-hoc analysis.

All statistical analysis was carried out using GraphPad Prism 6.0, significance was taken as $P<0.05$. Summary of analyses performed:

1) Effect of $MgSO_4$ vs. $MgCl_2$ - compared using t tests.

2) Tables 1 and 2- ANOVA with Bonferroni post hoc correction.

3) Comparison of $LogIC_{50}$ values for Magnesium on spontaneous or oxytocin contractions between gestational groups – ANOVA with Bonferroni post hoc correction.

4) Comparison of $LogIC_{50}$ values for effect of magnesium on spontaneous vs. oxytocin simulated contractions at each gestation - Extra sum of squares F-test.

3.4 Results

3.4.1 Control data and effects of Magnesium on late-pregnant mouse myometrium.

As there were little or no data concerning the actions of Magnesium on mouse myometrium, initial experiments were performed to, determine that Magnesium affected contractility, that the effects of $MgSO_4$ were due to Magnesium and not the anion, and to obtain an indicative concentration for concentration-response curves.

As shown in Figures 3.1A and 3.1B, stable, control contractions from pregnant mouse myometrium could be obtained for several hours without decrement in spontaneously active (A) and oxytocin-stimulated (B) tissues (typical of 9 preparations). Figure 1 also shows typical traces obtained using either 10 mM or 20 mM $MgSO_4$, (Figure 3.1C) or, $MgCl_2$ (Figure 3.1D), from three paired experiments (different animals), on oxytocin-stimulated pregnant myometrium. No significant differences were found between the effect of 10mM $MgSO_4$ and $MgCl_2$: amplitude: 70 $\pm$ 14% and 77 $\pm$ 9%; AUC: 50 $\pm$ 7 and 58 $\pm$ 2%, respectively, nor were any significant differences found between $MgSO_4$ or $MgCl_2$ during spontaneous activity (data not shown). Based on these data, the remaining experiments were performed using $MgSO_4$ as it is the sulphated form which is used clinically, and the effects of different concentrations and gestation were examined.

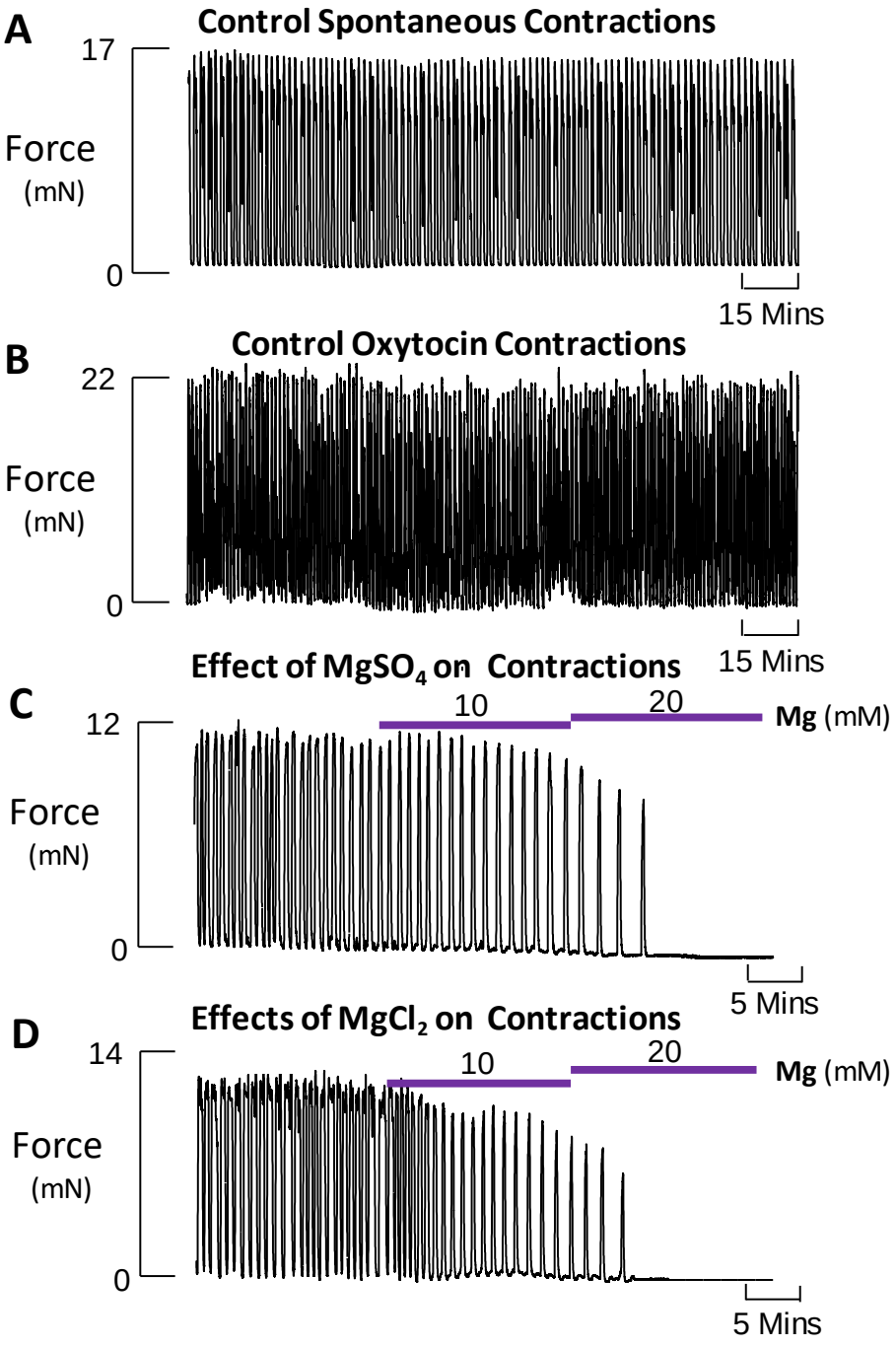


Figure 3.1 The effects of magnesium on contractions of mouse myometrium

Typical records showing contractions obtained from term-pregnant mouse myometrium. In this and all subsequent figures, data were obtained at 37°C and pH 7.4, with tissues superfused with physiological saline (containing 1. 2 mM MgSO4), in the absence (A & B) and presence of increased (10 or 20 mM) magnesium as sulphate (C) or chloride (D) salt. Traces B, C & D, were in the presence of oxytocin (0.5 nM).

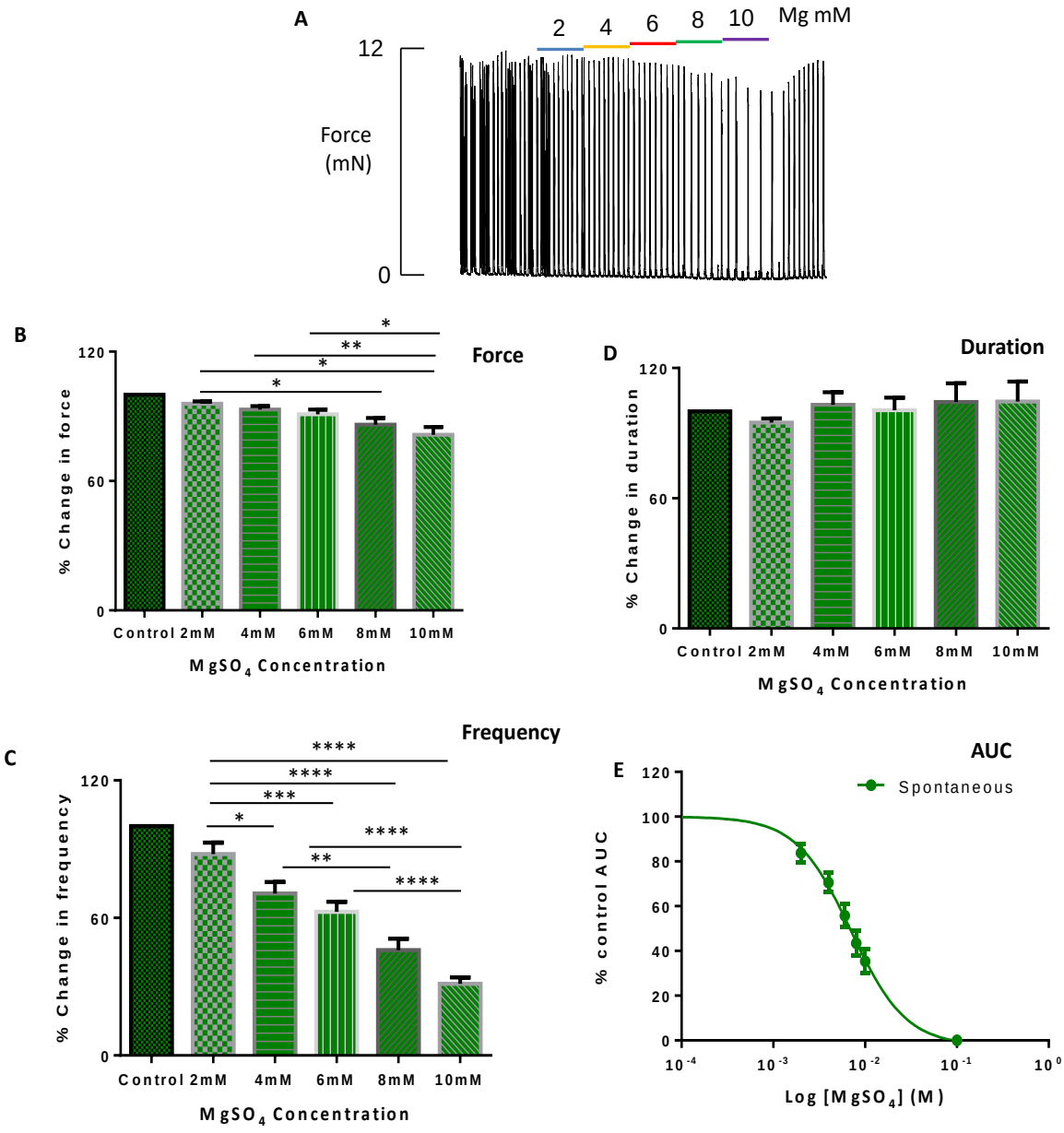


Figure 3.2: The effect of magnesium sulphate on spontaneous contractions of non-pregnant mouse myometrium.

Figure showing **(A)** representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in non-pregnant. Bar charts show the effect of magnesium sulphate on **(B),** amplitude, **(C)**, frequency (**D)** duration and **(E)** the area under the curve (AUC). Significant difference was found using ANOVA with Bonferroni post hoc test. The coloured bars indicate the 15-minute period when magnesium sulphate was added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), and 10mM (purple).

3.4.2 Effects of Magnesium on spontaneous contractions of non-pregnant myometrium.

The inhibitory effect of Magnesium on spontaneous contractions of non-pregnant mouse myometrium was examined. Increasing concentration of magnesium (2-10mM) was applied to spontaneously contracting myometrial strips for 15 minutes (Figure 3. 2A) and its effect on contractility parameters was determined (Figures 3. 2 B-E). In comparison to control, magnesium caused a concentration dependent decrease in force and frequency of contractions. There was a significant decrease of amplitude at 6mM and higher. At the highest concentration used, magnesium reduced amplitude of contractions to 81.5% ± 3.5 (relative to control, 100%), $P< 0.01$. At 4mM and higher, magnesium significantly reduced the frequency of contractions, reducing it to 31.2% ± 2.9 ($P<0.01$). There was no significant effect on duration of contractions even at the highest concentration. A concentration response curve was plotted to show the AUC of spontaneous contraction and the IC_{50} value was determined as 7.06mM ± 0.32.

3.4.3 Effects of Magnesium on spontaneous contractions of day 14 pregnant myometrium.

The concentration-dependent effect of magnesium on day 14 pregnant myometrium was examined. As described for non-pregnant myometrium, increasing concentrations of magnesium (2-10mM) was added to the strip (Figure 3.3A). Bar charts showing magnesium's effect on spontaneous contractions are represented in Figures 3.3B-E. Magnesium caused a significant reduction in contraction amplitude from 8mM onwards. At 10mM, magnesium reduced amplitude to 42.4% ± 12.5 (relative to control, 100%), $p < 0.001$. From 4mM onwards, the frequency of contractions was also significantly decreased. The duration of contractions was significantly decreased at 8mM and 10mM of magnesium's application. At 10mM, duration was reduced to 61.6% ± 13.6 ($p<0.05$) relative to control. From the concentration response curve plotted with AUC values, the IC_{50} was determined as 4.75mM ± 0.21.

3.4.4 Effects of Magnesium on spontaneous contractions of day 16 pregnant myometrium.

In day 16 pregnant mouse, $MgSO_4$ reduced spontaneous contractions in a concentration-dependent manner. Figure 3.4 shows a representative trace of magnesium's 15-minute application of each concentration. The bar charts showing magnesium's effect on amplitude, frequency and duration are represented in Figure 3.4B-E. Magnesium decreased contraction amplitude at 8mM and higher. At 10mM, contractions were reduced to 66.0% ± 15.0 ($P<0.01$). The frequency of contractions were also greatly reduced from 6mM onwards and reached 23.3% ± 11.7 at 10mM ($P<0.01$). There was no significant effect on the duration of spontaneous contractions. The IC_{50} value determined from AUC was 5.50mM ± 0.26.

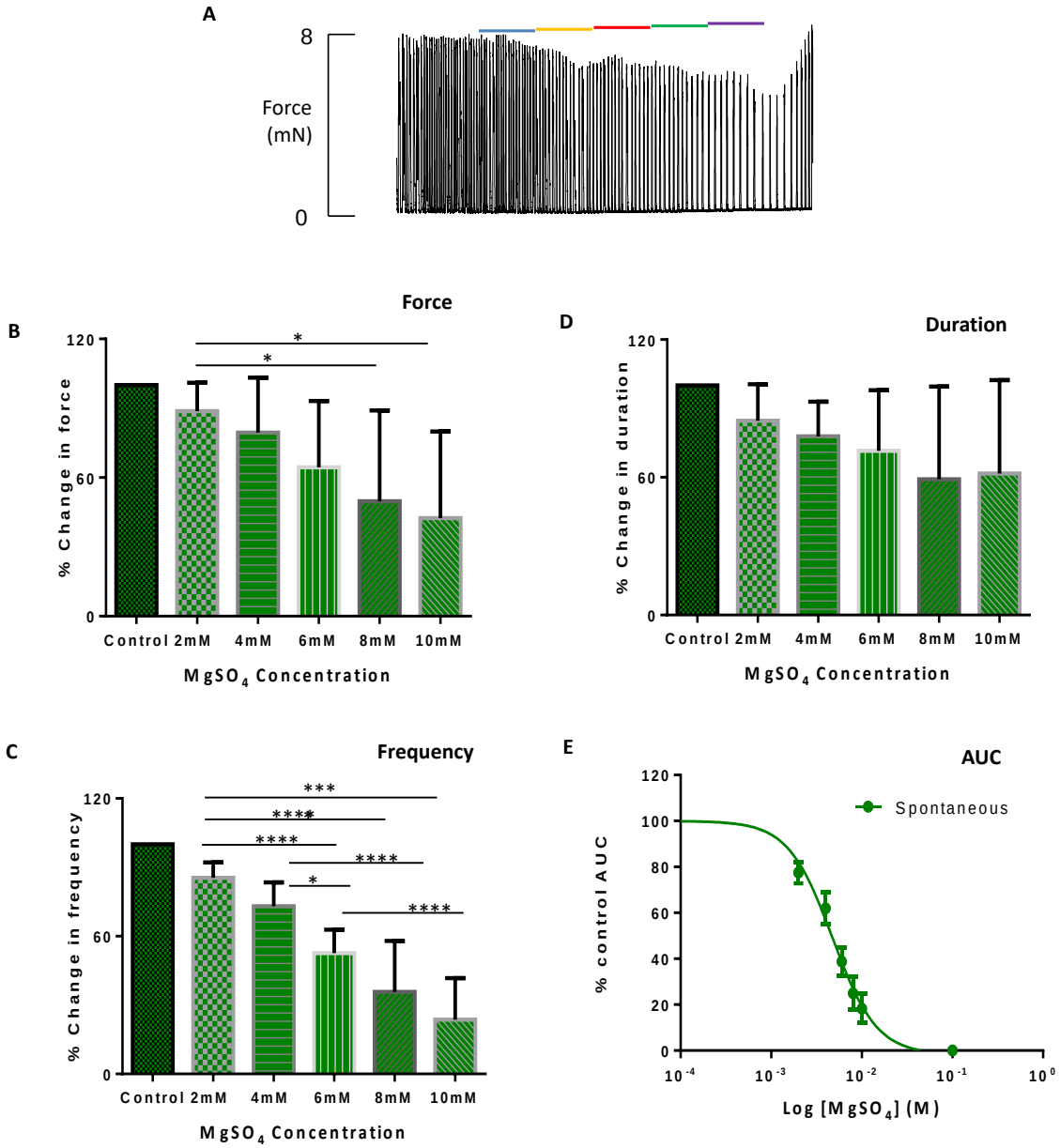


Figure 3.3: The Effect of Magnesium sulphate on spontaneous contractions of day 14 pregnant mouse myometrium

Figure showing (A) representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in non-pregnant.
Bar charts showing magnesium sulphate's effect on **(B),** amplitude **(C)**, frequency (**D),** duration and **(E)** the area under the curve (AUC).
Significant difference was found using ANOVA with Bonferroni post hoc test.
The coloured bars indicate the 15-minute period when magnesium sulphate was added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), and 10mM (purple).

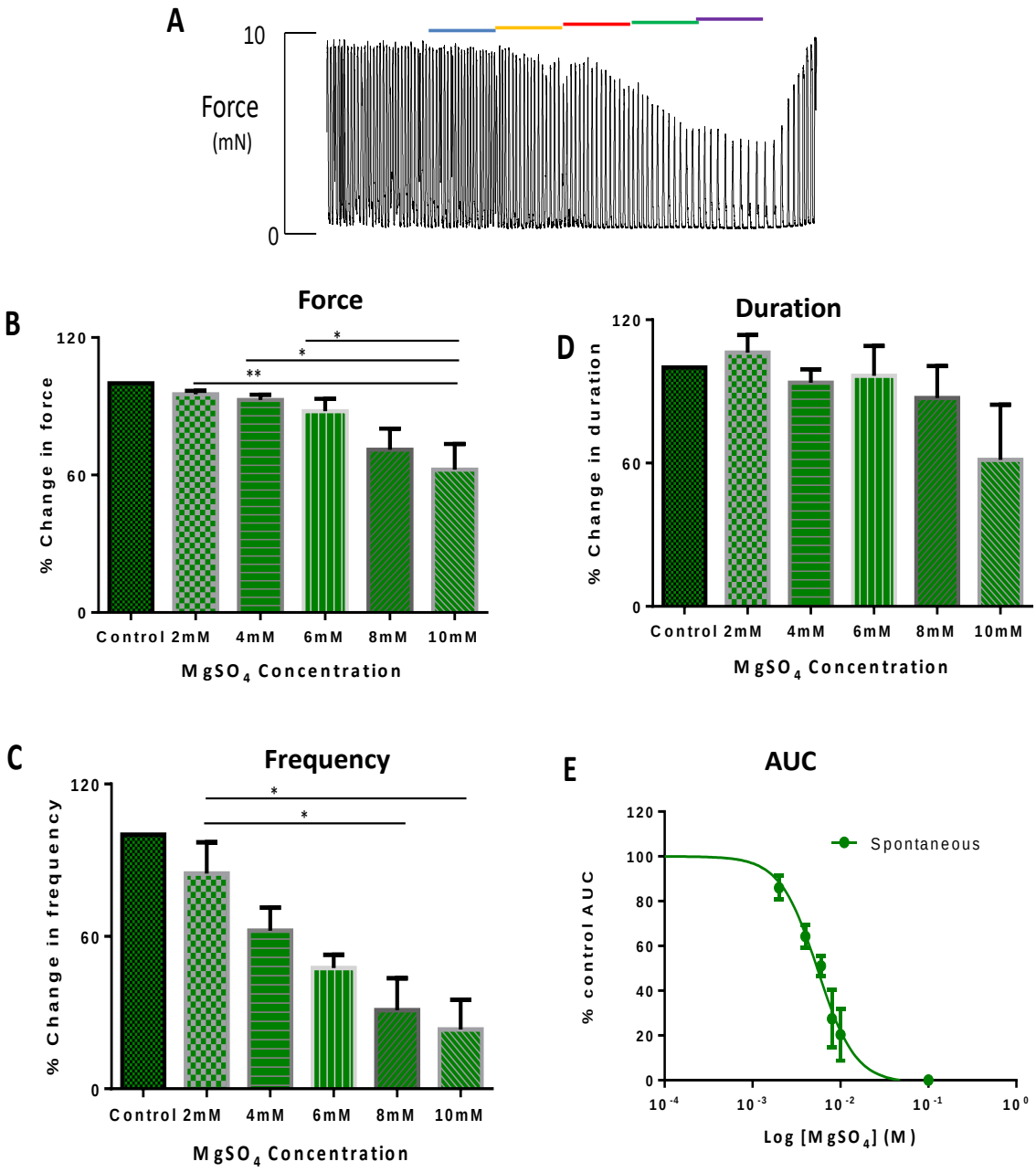


Figure 3. 4: The effect of magnesium sulphate on spontaneous contractions of day 16 pregnant mouse myometrium.

Figure showing (A) representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in non-pregnant. Bar charts showing magnesium sulphate's effect on **(B)** amplitude, **(C)**, frequency (**D)** duration and **(E)** the area under the curve (AUC).
Significant difference was found using ANOVA with Bonferroni post hoc test.
The coloured bars indicate the 15-minute period when magnesium sulphate was added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), and 10mM (purple).

3.4.5 Effects of Magnesium on spontaneous contractions of day 18 (term) pregnant myometrium.

Increasing concentrations of magnesium was added to spontaneously contracting term pregnant myometrium for 15 minutes (Figure 3.5A). Magnesium caused a concentration-dependent reduction and abolition of contractile activity (Figure 3.5B-D). Once magnesium was removed and perfused with normal PSS, contractions resumed. There was a significant decrease in contractile parameters- amplitude, and frequency of contractions when compared to control. The amplitude of spontaneous contraction was significantly reduced at concentration ≥ 4mM. The frequency of contractions was significantly reduced from the first application of magnesium (2mM). There was no significant effect on the duration of contractions. From the AUC values, a concentration-dependent curve was plotted (Figure 3.5E) and the IC_{50} value was determined as 3.08mM ± 0.16.

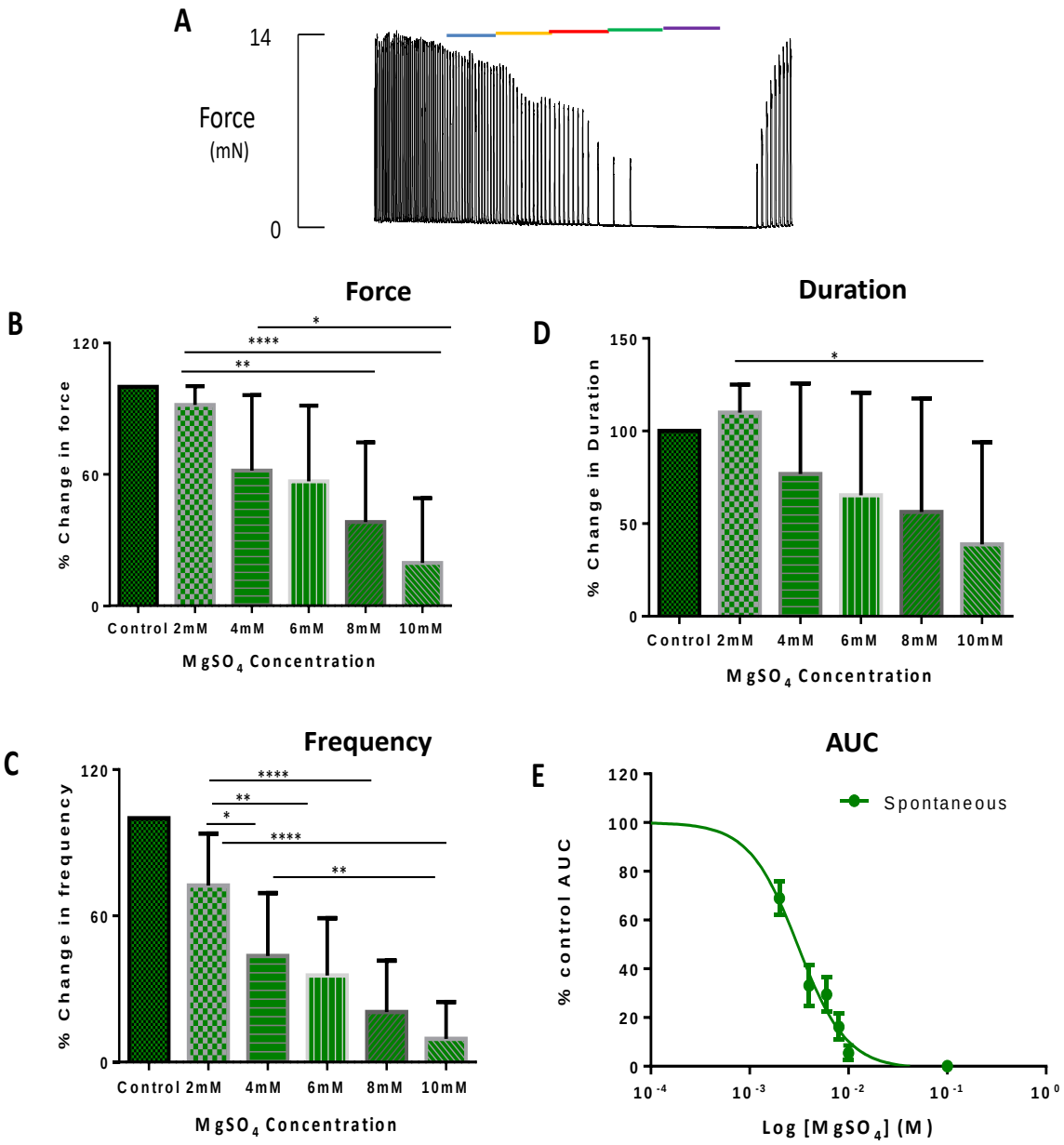


Figure 3. 5: The effect of magnesium sulphate on spontaneous contractions of day 18 pregnant mouse myometrium.

Figure showing (A) representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in non-pregnant. Bar chart showing magnesium sulphate's on **(B)** amplitude, **(C)**, frequency (**D)** duration and **(E)** the area under the curve (AUC). Significant difference was found using ANOVA with Bonferroni post hoc test.

The coloured bars indicate the 15-minute period when magnesium sulphate was added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), and 10mM (purple).

3.4.6 Comparing the effects of Magnesium on spontaneous contractions at different gestational states.

The effects of the application of increasing concentrations of $MgSO_4$ in the superfusate bathing the spontaneously contracting myometrial strips were examined and compared at day 14, 16 and 18 of pregnancy, and non-pregnant. Concentration-dependent inhibitory effects of Magnesium were found at each gestational state investigated as well as non-pregnant tissues. For all preparations, there was a decrease in frequency of contractions followed by a reduction in contraction amplitude. The mean data show that Magnesium reduces spontaneous activity of the myometrium in all tissue groups (Tables 3.1- 3.4), with its effect becoming more marked (and significant) as term approaches: In non-pregnant and day 14-pregnant tissues, contractions in the presence of 10mM $MgSO_4$ still persisted, with the AUC being 40% and 18% of control respectively. In term-pregnant myometrium 10mM $MgSO_4$ further and significantly reduced the AUC to negligible values (6%, Figure 3. 5A). The increased effects of Magnesium with gestation can also be appreciated from the fact that, in non-pregnant, day 14 and 16 tissues, contractions were abolished in just 1/22 preparations (on day 16), whereas at term, Magnesium abolished contractions in over half the tissues (6/11 preparations). In all preparations however, including those where contractions were abolished (e.g. Figure 3.5A), spontaneous contractions recovered to control values upon return to normal physiological saline ($MgSO_4$ 1.2mM).

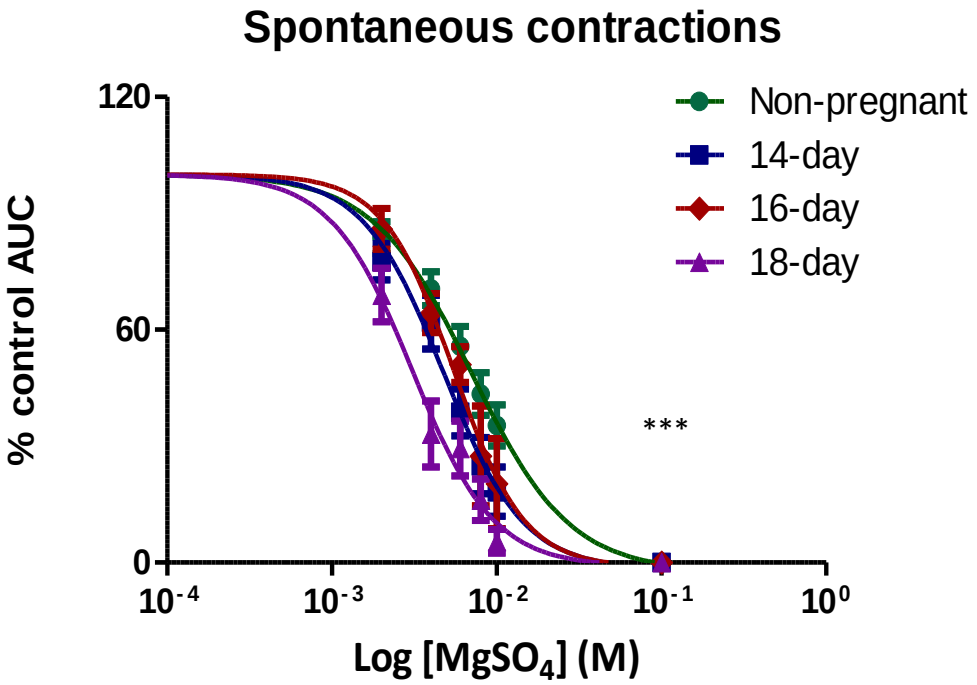


Figure 3. 6: The effects of magnesium sulphate on spontaneous contractions of mouse myometrium.

The concentration-response curves showing the effect of magnesium sulphate on force area under the curve (AUC) at different gestational states (non-pregnant: green circle, 14-day: blue square, 16-day: red diamond, term pregnant: purple triangle). The concentration-response curves significantly shifted to the left as gestation increased (P<.0001). Significant difference was found using ANOVA with Bonferroni post hoc test.

Plotting AUC concentration-response curves and calculating the IC_{50} values for Magnesium confirmed the mean data findings. The order of potency for $MgSO_4$ on spontaneous contractions was term-pregnant>mid-pregnant>non-pregnant, (Figure 3.6). The IC_{50} values fell from 7.06mM ± 0.32 (non-pregnant, n=10) to 4.75mM ± 0.21 (d 14, n=9) to 3.08mM ± 0.16 (term, n=11, $P<0.001$). There was no significant difference between the IC_{50} values for $MgSO_4$ at 14d and 16d gestation (4.75mM ± 0.21 and 5.50mM ± 0.26 respectively, $P>0.05$).

3.4.7 Effects of Magnesium on oxytocin-induced contractions of non-pregnant myometrium.

The effect of increasing concentrations (2-12mM) of magnesium on oxytocin-induced contractions of non-pregnant myometrium was examined (Figure 3.7A). 1nM oxytocin was added to PSS and applied to strips. Magnesium produced a concentration-dependent decrease in contractile parameters (Figures 3.7B-E). The amplitude of contractions was significantly inhibited at 10mM and higher. The frequency of contractions was significantly decreased at 4mM and higher. At 12mM, the frequency of contraction was reduced to 36.1% ± 4.1. There was no significant reduction in duration of contractions. The IC_{50} value from the concentration-response curve was determined as 8.07mM ± 0.40.

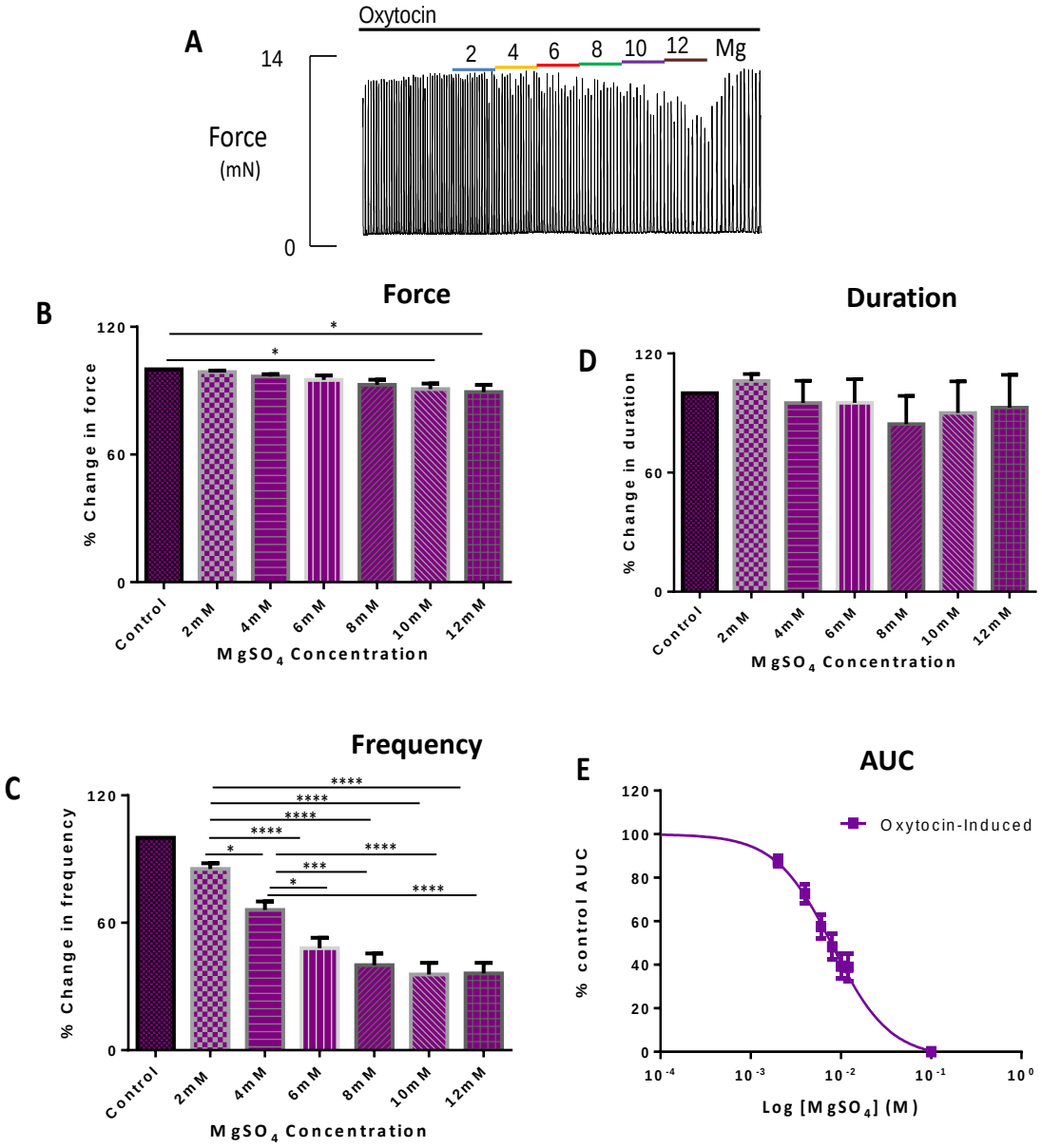


Figure 3. 7: The effects of magnesium sulphate on oxytocin-induced contractions of non-pregnant mouse myometrium.

Figure showing **(A)** representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in non-pregnant in the presence of 1nM oxytocin. Bar charts show the effect of magnesium sulphate on **(B)** amplitude force, **(C)**, frequency (**D)** duration and **(E)** the area under the curve (AUC). Increasing concentration of magnesium sulphate (2-12mM) caused gradual reduction in amplitude and frequency of contractions.
The short, coloured bars indicate the 15-minute period where magnesium sulphate was added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), 10mM (purple) and 12mM (brown).

3.4.8 Effects of Magnesium on oxytocin-induced contractions of day 14 pregnant myometrium.

Increasing concentrations of $MgSO_4$ (2-12mM) were added to oxytocin-induced contracting strips (Figure 3.8A). Magnesium produced a concentration dependent effect on oxytocin-induced contractions (Figure 3.8B-E) and its effect on contractile parameters was calculated. Magnesium significantly reduced amplitude at 12mM to 67.0 % ± 11.1 ($P<0.001$). The frequency of contractions was reduced at concentration ≥ 10mM. The duration was not significantly reduced. The IC_{50} value was determined as 10.25mM ± 0.24.

3.4.9 Effects of Magnesium on oxytocin-induced contractions of day 16 pregnant myometrium.

The response to $MgSO_4$ (2-12mM) when applied to contracting myometrium, in the presence of oxytocin (0.5nM), was a decrease in contractility (Figure 3.9 A-E). As reflected in Figure 3.9, compared to the control period, the amplitude was significantly decreased at ≥ 10mM. There was no significant decrease in the frequency and duration of contractions. The IC_{50} value for $MgSO_4$ in oxytocin-induced contractions, taken from the AUC concentration response curve, is 9.35m ± 0.38 (Figure 4.9 E).

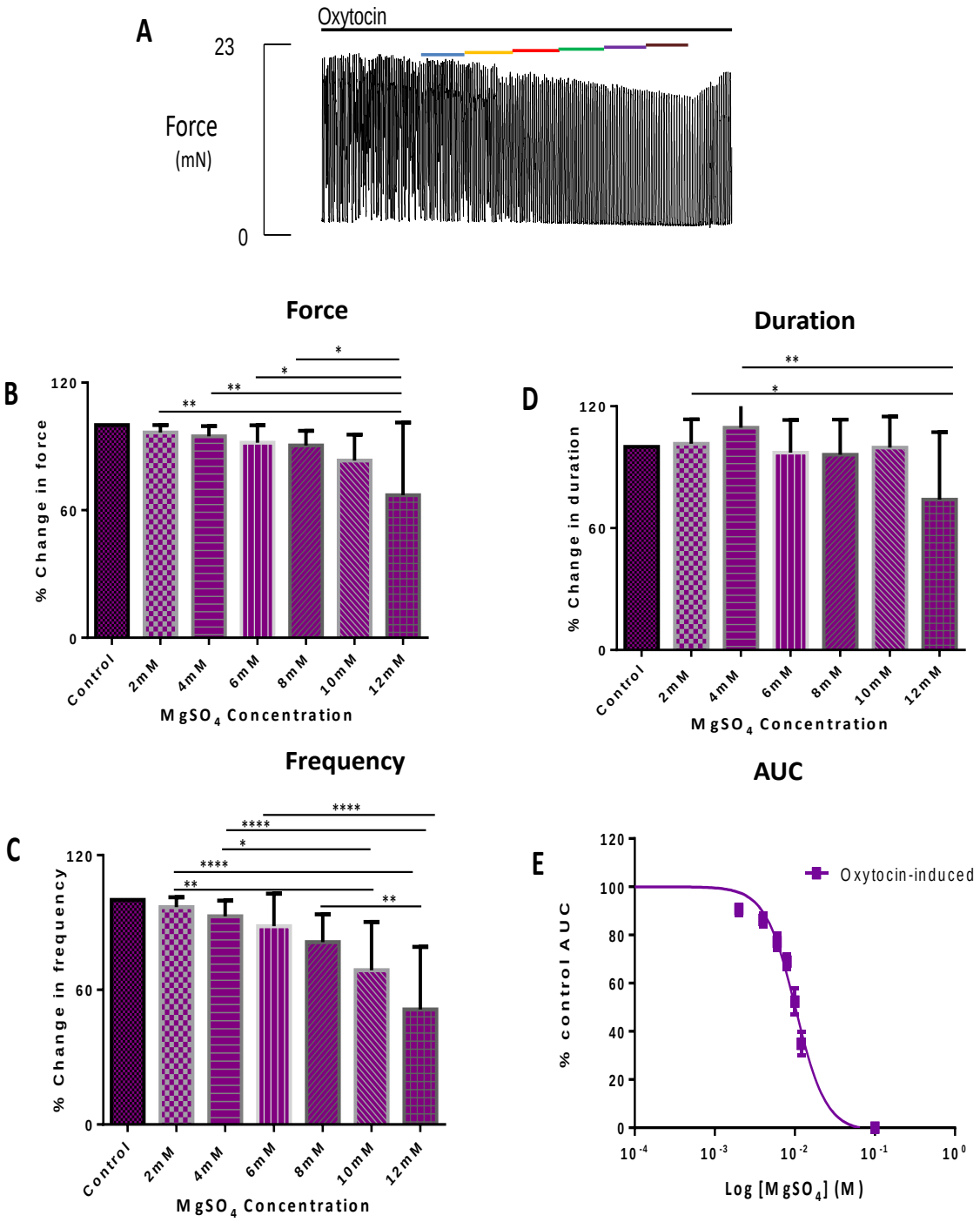


Figure 3. 8: The effects of magnesium sulphate on oxytocin-induced contractions of day 14 pregnant mouse myometrium

Figure showing (A) representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in day 14 pregnant mouse myometrium in the presence of 0.5nM oxytocin. Bar charts show the effect of magnesium sulphate on **(B)** amplitude, **(C)**, frequency (**D)** duration and **(E)** the area under the curve (AUC).

The short, coloured bars indicate the 15-minute period where magnesium sulphate was added. Increasing concentration of magnesium sulphate (2-12mM) caused gradual reduction in amplitude and frequency of contractions.

Increasing concentration of magnesium sulphate (2-12mM) caused gradual reduction in amplitude and frequency of contractions.

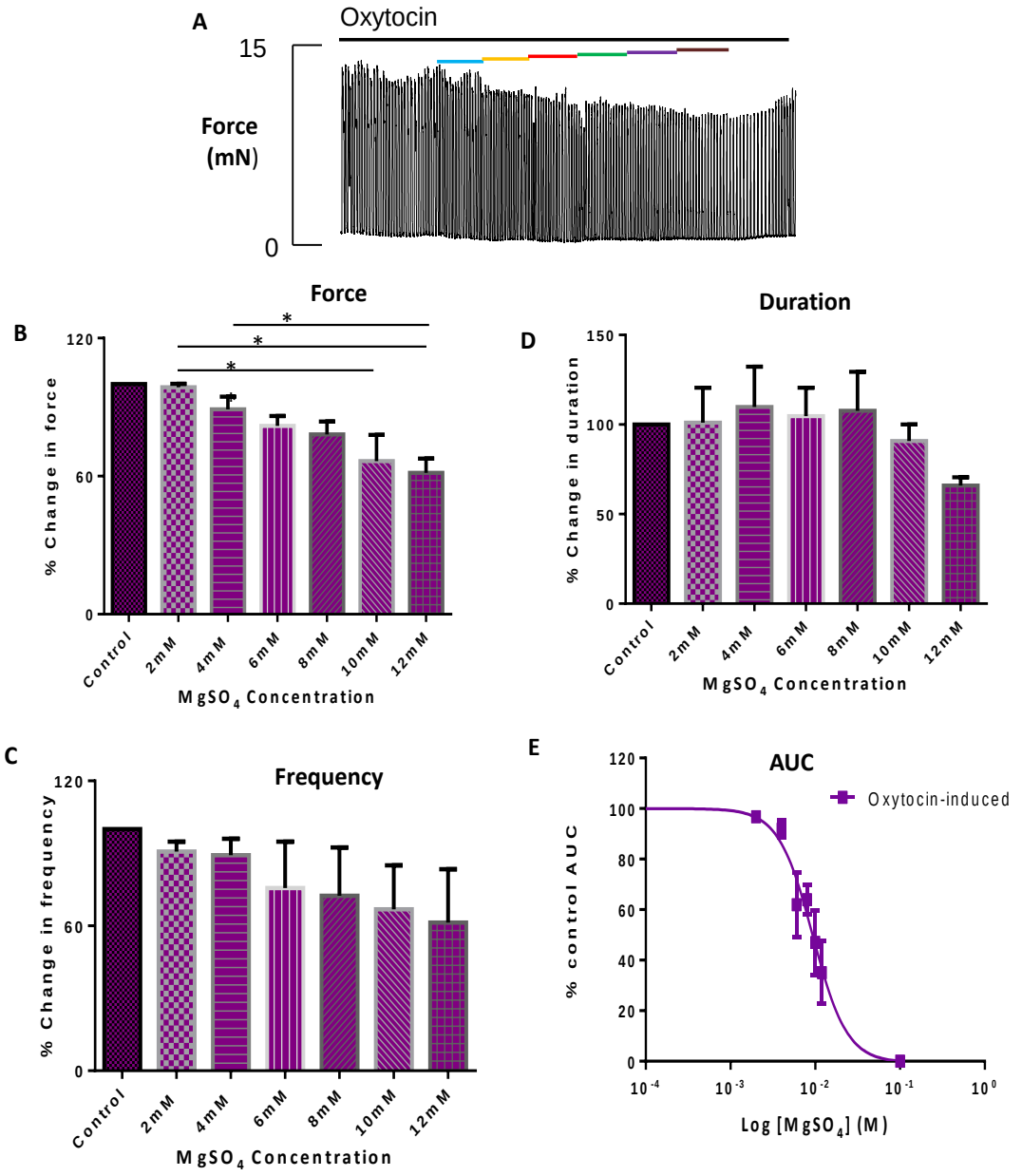


Figure 3. 9: The effects of magnesium sulphate on oxytocin-induced contractions of day 16 pregnant mouse myometrium.

Figure showing (A) representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in day 16 pregnant mouse myometrium in the presence of 0.5nM oxytocin. Bar charts show the effect of magnesium sulphate on **(B)** amplitude, **(C)**, frequency (**D)** duration and **(E)** the area under the curve (AUC).

The short, coloured bars indicate the 15-minute period where magnesium sulphate was added. Increasing concentration of magnesium sulphate (2-12mM) caused gradual reduction in amplitude and frequency of contractions.

3.4.10 Effects of Magnesium on oxytocin-induced contractions of day 18 pregnant myometrium.

In the presence of oxytocin, $MgSO_4$ had a concentration-dependent effect on day 18 pregnant tissue (**Figure 3.10A-E**). The amplitude of contraction was significantly reduced at 8mM and higher. At 12mM, magnesium reduced the contractions to 71.0 ±4.9 ($P<0.0001$). The frequency of contractions was significantly reduced at ≥ 6mM, reaching 58.0 ± 3.9 ($P<0.0001$). There was no significantly effect on the duration of contraction. The IC_{50} value from the AUC is determined as 9.75mM ± 0.21.

3.4.10 Comparing the effects of magnesium on oxytocin-induced contractions at different gestational stages.

Having found significant effects of Magnesium on spontaneous contractility I next investigated whether these effects were altered when the tissues were stimulated with oxytocin. Using the same protocol as for spontaneous contractions, the data obtained in the presence of oxytocin also showed a concentration-dependent decrease in all parameters of contraction. This was evident in all tissue groups. Representative traces showing the effect of $MgSO_4$ in the presence of oxytocin are given in Figures 3.3A-D.

Unlike spontaneous activity, in the presence of oxytocin, the myometrium was still producing significant amounts of force at 10mM Magnesium in all preparations. In order therefore to accurately plot concentration-response curves, assist with curve fitting and better understand the response of the tissue, Magnesium was increased to 12 and 20 mM. Typical responses to 20mM Magnesium are shown in Figure 3.1C.

As is clear in the example traces in Figure 3.3, for all pregnant tissues, a less potent inhibition with Magnesium was found in the presence of oxytocin compared to its application to spontaneous contractions, i.e. without oxytocin (Figure 3.2).

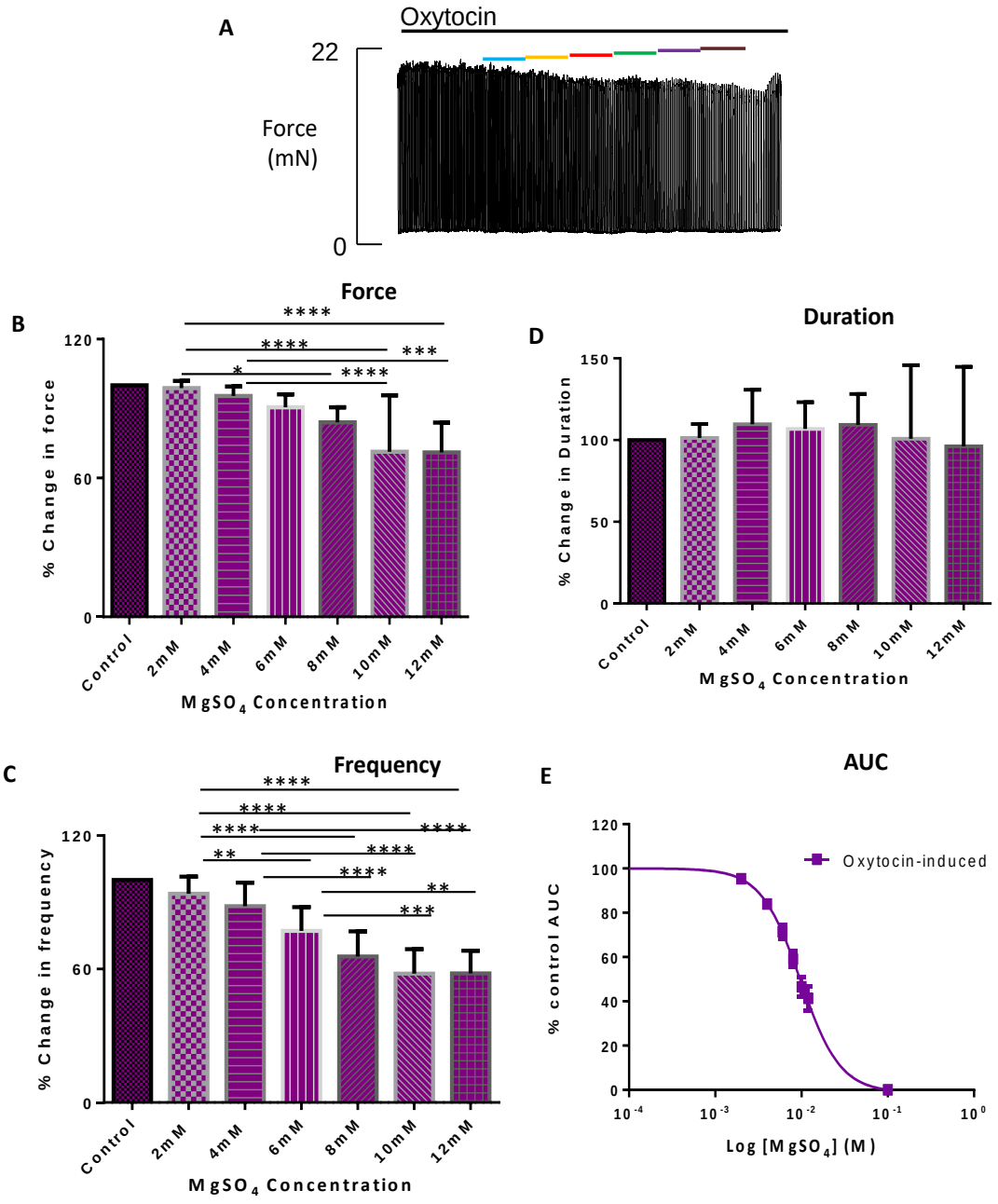


Figure 3. 10: The effects of magnesium sulphate on oxytocin-induced contractions of day 18 pregnant mouse myometrium

Figure showing (A) representative isometric trace showing the effect of increasing concentrations of magnesium sulphate in day 18 pregnant mouse myometrium in the presence of 0.5nM oxytocin. Bar charts show the effect of magnesium sulphate on **(B)** amplitude, **(C)**, frequency (**D)** duration and **(E)** the area under the curve (AUC). The short, coloured bars indicate the 15-minute period where magnesium sulphate was added. Increasing concentration of magnesium sulphate (2-12mM) caused gradual reduction in amplitude and frequency of contractions.

Additionally, in pregnant tissues, concentration-response curves for Magnesium in the presence of oxytocin were also shifted to the right compared to spontaneous contractions (Figure 3E and 4B-D), resulting in significantly greater IC_{50} values with oxytocin stimulation. The greatest shift in IC_{50} values was seen for term-pregnant tissues from 3.08mM ± 0.16 in spontaneous conditions to 9.75mM ± 0.21 with oxytocin ($P<0.0001$). A significant shift in IC_{50} values was also seen for day 14 gestation tissues from 4.75mM ± 0.21 in spontaneous conditions to 10.25mM ± 0.24 in oxytocin ($P<0.0001$) and day 16 gestation tissues from 5.50mM ± 0.26 in spontaneous conditions to 9.35m ± 0.38 in oxytocin ($P=0.0031$). In contrast, in non-pregnant tissues, the presence of oxytocin (even at 1nM) did not significantly alter the IC_{50} values for Magnesium: 7.06mM ± 0.32 in spontaneous to 8.07mM ± 0.40 in the presence of oxytocin (Figure 3.4A, $P=0.3236$), hence in non-pregnant tissues, Magnesium is equipotent in the presence and absence of oxytocin.

My data showed that, a consequence of oxytocin significantly increasing the IC_{50} values in all pregnant tissues was that there were no longer any gestational differences between them, in the effects of Magnesium (Figure 3.4). In other words, there was no significant difference in the inhibitory effect of Magnesium as pregnancy progressed, unlike what had been found above for spontaneous conditions. Instead, in the presence of oxytocin, the potency of $MgSO_4$ was reduced such that it was similar to its potency in non-pregnant tissues irrespective of pregnancy.

Tables showing the mean values ± SEM for all results in this chapter are in the appendix section.

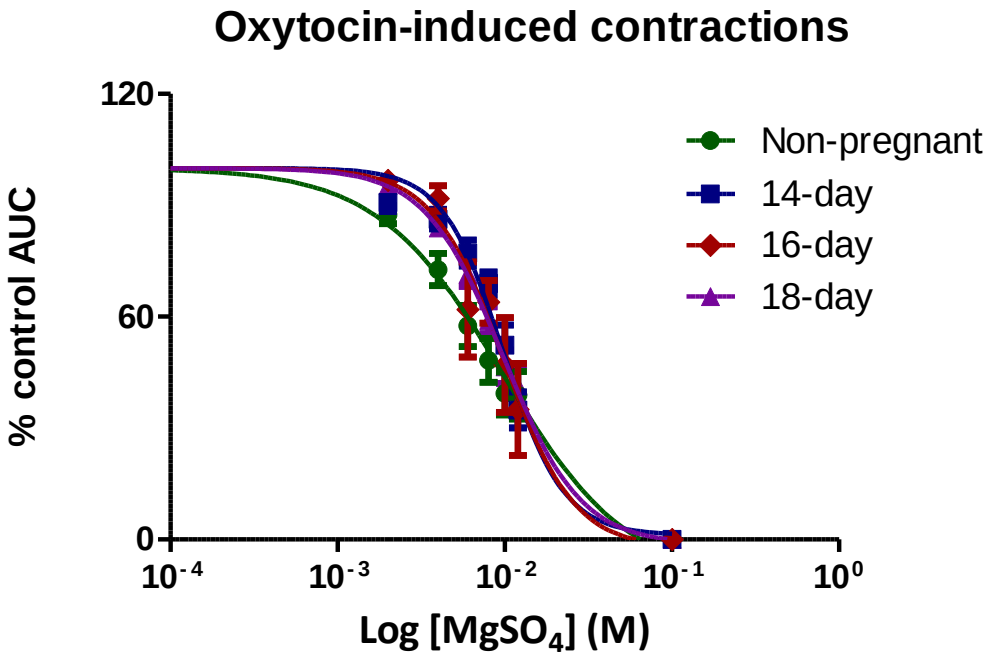


Figure 3. 11: The effects of magnesium sulphate on oxytocin-induced contractions of mouse myometrium.

The concentration-response curves show the effect of magnesium sulphate on AUC of contraction at different gestational states (non-pregnant : green circle, 14-day: blue square, 16-day: red diamond, term-pregnant: purple triangle). There was no significant difference between the concentration-response curves for the different gestational states, determined using ANOVA with Bonferroni post hoc test.

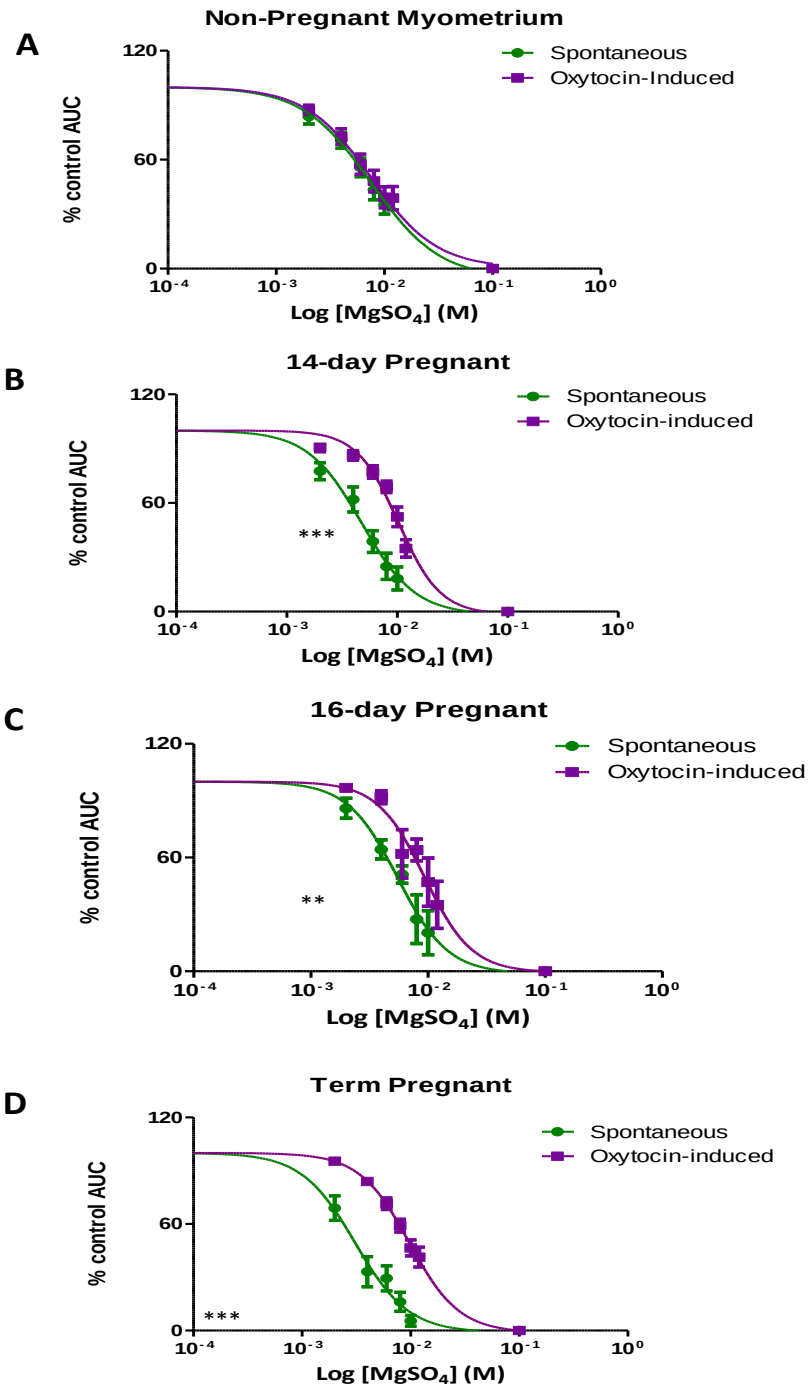


Figure 3. 12: The effect of magnesium sulphate between spontaneous and oxytocin-induced contractions at different gestational states.

The concentration-response curves show the effect of magnesium sulphate on force integral (AUC) of contraction in spontaneous (green circles) and oxytocin-induced contractions (purple squares). There was no significant difference (P=0.062) between the concentration-response curves of non-pregnant myometrium **(A)**. For 14-day **(B)**, 16-day **(C)** and term-pregnant myometrium **(D)**, the concentration-response curves were significantly shifted to the right in the presence of oxytocin (P<.0001, P<0.01 and P<0.0001 respectively). Significant difference in activity was determined using F-test.

3.4 Discussion

Magnesium has been reported to suppress myometrial contractions and for this reason has been used over the last five decades in the treatment of preterm labour. Many clinical studies however, including Cochrane reviews (Crowther et al., 2014), conclude or suggest that $MgSO_4$ is ineffective at delaying labour. Thus, the clinical use of $MgSO_4$ in the treatment of preterm labour is questioned (Grimes and Nanda, 2006, Hacivelioglu et al., 2007, Keirse, 2003b), highlighting the need for further studies. This study was conducted to better understand the effects of Magnesium on uterine contractility. It was designed to examine if physiological changes, namely gestational state and hormonal background, could influence myometrial responses to Magnesium. In this way, a better understanding of the disappointing clinical findings will be obtained and perhaps suggest a more stratified approach to its use as a tocolytic, to help prevent preterm labour. Magnesium's effects as a tocolytic may synergise with its use and action to treat eclampsia. This data provide fresh insights, as they show that preterm myometrium is much less sensitive to the relaxant effects of Magnesium than term myometrium, which could explain its lack of clinical efficacy. If oxytocin was present, its efficacy was further decreased, at all gestational stages. Thus, taken together I conclude that Magnesium's actions are influenced by both gestational state and hormones, such that, at least in mice, it is least effective in early gestation with oxytocin present and most effective at term in the absence of oxytocin.

In vitro Mouse myometrium

This study was conducted on mouse myometrium so that myometrial preparations at several stages of gestation as well as non-pregnant tissue could be obtained. Myometrium from pregnant mouse has previously been reported to produce rhythmic spontaneous contractions *in vitro* for many hours(Matthew et al., 2004b). Consistent with this, I found all the uterine strips generated spontaneous contractions which were stable within a period of 45 -60 minutes, and remained regular without significant reduction in amplitude or frequency for many hours. This

therefore allowed the effect of incremental concentrations of Magnesium to be examined and concentration-response curves to be fitted. There has been just one previous report of Magnesium's actions on mouse myometrium, in a lipopolysaccharide model of preterm birth (Sugawara et al., 2007). These authors only studied mid-gestation (14 day) uterus and reported inhibition of spontaneous contractions with Magnesium; an effect which was decreased by lipopolysaccharide. The findings of an inhibitory effect on term-and non-pregnant myometrium are consistent with findings in other species(Phillippe, 1998, Arrowsmith et al., 2016, Onwochei et al., 2017, Tica et al., 2007, Kantas et al., 2002) including humans. Future studies should attempt to obtain human biopsies at different stages of gestation to confirm these findings, although obtaining preterm biopsies is challenging. In addition, to reflect the somewhat heightened state of contraction that may be associated with preterm labour compared to the non-labouring strips used here, a preterm labour mouse model, such as that induced by LPS or other agents, could be used to investigate the *in vivo* therapeutic effect of $MgSO_4$.

***Magnesium's mechanism of action in myometrium*.**

The effect of $MgSO_4$ on contractions at different gestational stages was investigated and my data shows that regardless of gestational age or pregnancy status, it can reduce myometrial contractions. This inhibitory effect was reversible and the prompt recovery after washout suggests no ill effects of Magnesium on myometrium, even at high concentrations. The concentrations of $MgSO_4$ used by us *in vitro* were empirically determined, and in the case of oxytocin-evoked contractions, going in to pharmacological rather than physiological concentrations, to enable maximal effects to be obtained.

It is unlikely that intracellular Magnesium will have risen during the course of the experimental protocol, due to the activity of Na^+ - Mg^{2+} exchangers and intracellular buffers(Fomin et al., 2006), as well as Magnesium's slow penetrability through cell membranes (Martin et al., 1987). That the effects of Magnesium on the myometrium are relatively rapid and reversible, suggests that its main mechanism

of action is likely to be extracellular. The rapid reversal of Magnesium's effect is clinically useful, for example if delivery is by cesarean section, as it reduces the risk of postpartum haemorrhage (Lau et al., 2001). Magnesium use in preeclampsia has been associated with increased post-partum haemorrhage in some studies (Szal et al., 1999), but in another study, its use was not associated with additional blood loss (Graham et al., 2016).

The major mechanism of Magnesium's action to relax uterine smooth muscle is, as with other excitable tissues, due to its cationic competition with Calcium (Phillippe, 1998). In myometrium, contractions, whether spontaneous or agonist induced, are critically dependent upon Calcium entry through L-type Calcium channels (Wray et al., 2001, Wray et al., 2003). Thus when increased Magnesium competes with Calcium, entry of Calcium falls and hence contractions reduce and can even be abolished, as our group and others have shown (Popper et al., 1989). The fall in intracellular Calcium will also lead to a fall in the Calcium content of the sarcoplasmic reticulum (SR)(Shmigol et al., 2001). This reduced SR Calcium available when agonists such as oxytocin produce IP_3, will reduce their ability, to increase myometrial contractility(Phillippe, 1998). In this way, Magnesium will be expected to reduce the force of spontaneous and oxytocin-induced contractions. Fomin et al., (2006) showed that $MgSO_4$ reduced spontaneous, oxytocin- and KCl-induced myometrial contractility and all were associated with a decrease in intracellular Calcium (Fomin et al., 2006). In addition, they found no shift in the force-Ca relationship in the myometrium. Together this provides strong evidence that Magnesium's effects are predominantly extracellular and on Calcium entry.

At all gestations and in non-pregnant tissue, my data show a significant reduction in force and frequency of contractions with addition of $MgSO_4$. A reduction in force is most likely because of Magnesium's antagonistic effect with Calcium at L-type calcium channels, as discussed above. Frequency of contractions is mainly dependent on excitability and membrane potential, hence a reduction in frequency suggests the pacemaker activity may also be affected by $MgSO_4$ (Kawarabayashi et al., 1989). For example, should differences in the resting membrane potential of some pacemaker cells exist, it may make them more susceptible to the effects of

Magnesium leading to fewer action potentials being triggered (Wray et al., 2015). The figures throughout show that a decrease in the frequency of contractions, usually preceded a fall in force. This finding is consistent with observations on human myometrium (Tica et al., 2007, Tang et al., 2014). Additionally, although not yet tested in uterus, Magnesium may affect inter-cellular coupling such as via gap junctions (Matsuda et al., 2010, Rimkute et al., 2018), hence reducing the likelihood of frequent, synchronous contractions being produced.

Gestational effects

In spontaneous contractions, the effect of $MgSO_4$ was most potent at term, and least potent in non-pregnant tissues. The latter group were not staged for oestrous and should therefore be treated as a mean of any cyclical, oestrous changes (Dodds et al., 2015, Wray and Noble, 2008). I found that even on day 16 of gestation, Magnesium's effect on the myometrium was significantly less than at term, suggesting that the changes in sensitivity are on-going throughout pregnancy. Given that the underlying mechanism of Magnesium's effects is via decreasing excitability and Calcium entry, then these findings point to a difference in the Calcium channels with gestation. There have been surprisingly few studies of L-type Calcium expression with gestation in the myometrium. The work that has been done suggests an increase in expression and changes of subunits from mid-gestation onwards. Tezuka et al (1995) found a marked increase in α1 expression in pregnancy, especially in the last half of gestation until term, followed by a decrease during labour (Tezuka et al., 1995). Similarly, Mershon *et al* (1994) studied the expression of α_1 subunit and showed a gradual increase in the mRNA towards term followed by a decrease during parturition (Mershon et al., 1994). They also showed an increase in the number of dihydropyridine binding sites (markers for L-type channels) in the last half of gestation. Thus, a simple effect on Calcium entry is difficult to propose to explain gestational differences, unless the switch to increased α-1 subunits confers an increased susceptibility to Magnesium blockade. Rather, my data suggests that at term the effect of Magnesium is enough to reduce

excitability and make action potential firing less likely, as occurs with its use in neuronal tissues to protect the brain.

Oxytocin and Magnesium

To determine if $MgSO_4$ can affect contractile parameters in the presence of an agonist, its effect on oxytocin-evoked contractions was determined. Oxytocin was used due to its particular importance to labour. Oxytocin can directly and indirectly stimulate myometrial contractions (Arrowsmith and Wray, 2014). This data show that a greater concentration of Magnesium is needed to inhibit oxytocin-stimulated contractions compared to spontaneous contractions in pregnant tissues. This is in agreement with data from other studies including human myometrium (Arrowsmith et al., 2016, Onwochei et al., 2017). However, oxytocin did not significantly change the IC_{50} for Magnesium in non-pregnant tissues, from that found for spontaneous contractions. We found this to be the case even when oxytocin was used at 1nM i.e. double the oxytocin concentration used in pregnant tissues. There was no difference in the potency of $MgSO_4$ on oxytocin-stimulated contractions between the different gestational groups. The greatest shift in Magnesium's potency with oxytocin was observed for term-pregnant myometrium; this shifted the IC_{50} from around 3 mM in spontaneous conditions to almost 10 mM. In terms of clinical applications, the effect of oxytocin is to shift the concentration from therapeutic to lethal. Therapeutic concentrations of Magnesium are reported at 2.5 mM (5 mEq/L) or below (Tica et al., 2007), and for seizure management in preeclampsia, 3 mM (Onwochei et al., 2017).

Although oxytocin acts via several mechanisms, an important mechanism is increasing membrane potential (resulting in opening of the L-type Ca^{2+} channels and increased Calcium entry), releasing Calcium from the sarcoplasmic reticulum and preventing Calcium exit (Arrowsmith and Wray, 2014). These mechanisms will counteract Magnesium's actions and may explain why a greater concentration of $MgSO_4$ is needed to inhibit oxytocin induced contractions close to term. The lipid environment around the oxytocin receptor influences its affinity for oxytocin (Arrowsmith and Wray, 2014, Gimpl and Fahrenholz, 2001, Noble et al., 2006) , an

effect attributed to its partitioning into lipid rafts and their effect on signal transduction (Smith et al., 2005, Draeger et al., 2005). It was also reported that as well as high cholesterol, the high affinity form of the oxytocin receptor requires Magnesium, working as an allosteric modulator. Thus, when Magnesium is increased it may increase the oxytocin signalling and further counter the relaxant effect of Magnesium on Calcium entry.

Transition to labour in humans is associated with increased oxytocin receptor expression (Kimura et al., 1992), which increases the sensitivity of the uterus towards oxytocin (Kubota et al., 1996). An increase in oxytocin receptor expression towards term in mouse, has also been shown (Kubota et al., 1996). Consistent with this is the finding that little, if any, contractility stimulation to 0.5nM oxytocin was observed in non-pregnant tissues and hence the use of 1nM, to assess the effect of oxytocin on the potency of $MgSO_4$ (control data, not shown). Although not accounting for the lack of gestational differences, the increased drive on contraction produced by oxytocin, and the increased number of receptors, and increased affinity, may also partly explain why a greater concentration of $MgSO_4$ is needed to inhibit contractions in its presence.

3.5 Study Limitations

Although, as indicated above and in chapter 1, there is much that is similar between mouse and human, nevertheless the effect of magnesium should be further explored in human tissues.

This study did not use strips from labouring mouse tissues as they were much more difficult to obtain. Studies testing magnesium on labouring tissues may prove helpful.

I have focussed on the most plausible mechanisms of magnesium's tocolytic effect, based on the literature and our understanding of EC coupling. Other mechanisms however may have operated in this model, such as Calcium-independent pathways which may have additionally been affected by elevated Magnesium. However, the role of Ca-sensitisation in the mouse uterus is thought to be minor (Wray, 2007). However, this work, and studies by others have not considered possible effects of

magnesium on potassium channels. Finally, *in vitro* studies will always be a step removed from in vivo conditions, although I optimised the experimental conditions to near-physiological, in vivo conditions to aid translation.

3.6 Conclusion

This *in vitro* study shows that $MgSO_4$, acting extracellularly, concentration-dependently inhibits spontaneous and oxytocin-induced myometrial contractions in both the pregnant and non-pregnant mouse, with greater efficacy observed in term-pregnant tissues. However, oxytocin decreases the potency of $MgSO_4$ in pregnant tissues, due to its stimulation of contraction and perhaps due to Magnesium allosterically increasing the affinity of the oxytocin receptor, which may underlie its lack of efficacy as a relaxant i.e. tocolytic, *in vivo*.

Chapter 4

The Effect of Combining Magnesium with Other Tocolytics

Chapter 4

The Effect of Combining Magnesium with Other Tocolytics

4.1 Abstract

Several uterine tocolytics have been investigated in the treatment of preterm labour: oxytocin receptor antagonist e.g. atosiban, calcium channel blockers, e.g. nifedipine, prostaglandin synthetase inhibitor e.g. indomethacin, β- adrenoceptor agonist e.g. ritodrine and Magnesium sulphate ($MgSO_4$). Studies have shown tocolytics to only delay labour for 48 hours and at best 7 days. They however delay labour long enough for administration of corticosteroids. $MgSO_4$ though not a tocolytic used in the UK is administered in threatened preterm birth cases (<30 weeks). Since these tocolytic agents act via different pathways, I hypothesized that a combination of $MgSO_4$ with other tocolytics may produce a greater inhibitory effect than the individual drugs used alone. The aim of the study was to investigate the dual effect of magnesium (Mg) plus other tocolytics *in vitro.*

Longitudinal uterine strips were obtained from term pregnant C57BL/6J mice (19 days gestation). After spontaneous contractile activity reached a steady state, 0.5nM oxytocin was added. Contractile activity was recorded using digital software (Labtrax). The concentration-dependent effect of indomethacin, atosiban and nifedipine was examined with the IC_{50} value (concentration at which there is a 50% reduction) determined for each drug. Magnesium sulphate was then applied either alone or in combination with (i) 3 µM (spontaneous) and 30 µM (oxytocin) indomethacin, (ii) 500 nM atosiban or (iii) 0.03 nM nifedipine at their predetermined IC_{50} values. Control contractile activity and activity after addition of each combination were measured using the last 10 minutes of activity and the changes expressed as a percentage of control (100%).

All tocolytics investigated (Mg, indomethacin, atosiban and nifedipine) inhibited myometrial contractions when used alone. In spontaneous contractions, Mg + indomethacin reduced the area under the curve (AUC) of contractions to 4.6% ± 3.1,

compared to 62.7% ± 8.0 with indomethacin alone (P<0.001). With the addition of oxytocin, the efficacy of Mg plus indomethacin was reversed. The combination reduced AUC of contractions to 70.6% ± 3.1 compared to 38.4% ± 5.8 with indomethacin alone (P<0.01). The combination of Mg plus Atosiban (n=8) greatly reduced AUC of oxytocin-induced contractions to 1.3% ± 1.0 compared to 56.2% ± 7.0 with atosiban alone (P<0.0001). In spontaneous contractions, Mg plus nifedipine (n=7) reduced the AUC to 46.4% ± 6.7. Similarly in oxytocin-induced experiments, Mg + nifedipine (n=6) reduced AUC of contractions to

This *in vitro* data suggest that Mg plus atosiban produced a greater inhibitory effect than either the drugs alone or the other combinations.

4.2 Introduction

Preterm birth is defined as birth before 37 weeks of pregnancy. Globally, preterm births affect almost 15 million people yearly and currently accounts for about 11.1% of all births (Howson et al., 2013). It is generally thought that about 65 to 70% of preterm births are spontaneous which could either be due to spontaneous preterm labour or births following a premature rupture of membranes (Goldenberg et al., 2008). The precise cause of spontaneous preterm labour is unknown; however, it could result from a range of factors and varies with gestational stages (Carvajal et al., 2017).These factors could include history of preterm birth, maternal age, race, urinary tract infections, poor nutrition, etc. (Goldenberg et al., 2008, Vogel et al., 2014b).

A wide range of drugs are used to suppress or reduce uterine contractions, known as tocolytics. Since the discovery of the first tocolytic, relaxin, many other tocolytics have been developed. One major factor that contributed to the development of tocolytics was to improve perinatal mortality (Keirse, 2003a). However, with the development of these drugs, an actual reduction in preterm birth has not been evident and there hasn't been an improvement in infant outcome following tocolytic therapy (Keirse, 2003a). Even though the administration of tocolytics prolongs labour, several studies have also shown that they are not without their side effects which may be foetal or maternal (Olson et al., 2008) (de Heus et al., 2009).

As detailed in chapter 3, uterine contractions is regulated by the increase in intracellular Ca^{2+} in the myometrium. Ca^{2+} binds to calmodulin, activating myosin light chain kinase (MLCK), and resulting in the phosphorylation of myosin light chains and initiation of cross-bridge cycling in contractile proteins (Gáspár and Hajagos-Tóth, 2013, Wray et al., 2003). Calcium influx is therefore a major pathway for myometrial contraction. Agonists (e.g. oxytocin and prostaglandins) by binding to their receptors result in augmentation or stimulation of the pathway (Arrowsmith et al., 2010). These agonists could also initiate other intracellular mechanisms which may influence force. Oxytocin acts by activating OTRs, causing

the activation of phospholipase C-β (PLCβ), which controls hydrolyses of phosphatidylinositol bisphosphate (PIP_2) leading to the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). Intracellular calcium is then mobilized from the SR leading to stimulation of the uterus. Besides this mechanism, there are other more complex pathways via which oxytocin acts. Prostaglandins, which act by binding to different G-protein coupled produces different cascades of intracellular pathways as a result of each of them having individual receptors. This stimulation causes an increase in free intracellular calcium concentration in myometrial cell. There is evidence that stimulation with oxytocin results in an upregulation of cyclooxygenase-2 (COX-2) and prostaglandin synthesis in other uterine tissues (e.g. epithelium and amnion), (Chibbar et al., 1993, Wilson et al., 1988, Terzidou et al., 2011). Oxytocin also increases COX-1 and COX-2 activity leading to PGE2 formation (Blanks and Thornton, 2003).

Tocolytics, however, cause uterine inhibition or relaxation by acting on these contractile pathways (Figure 4.1). The different classes of tocolytics: magnesium sulphate, oxytocin receptor antagonists (e.g. atosiban), prostaglandin inhibitor (e.g. indomethacin), calcium channel blockers (e.g. nifedipine), beta-adrenergic receptor (AR) agonists (e.g. ritodrine) all have a unique mechanism of action. Magnesium acts both extra- and intracellularly causing decreased Ca influx and Ca release from the SR (Arrowsmith et al., 2010). It acts mainly by antagonising Ca entry at the L-type Ca channel thereby decreasing intracellular calcium concentration. Atosiban is the only selective oxytocin receptor antagonist currently used in clinical practice (Hubinont and Debieve, 2011) although there have been others developed including barusiban. Atosiban acts by competing with oxytocin from binding to its receptors in the myometrium and decidua (Mattison, 2013).

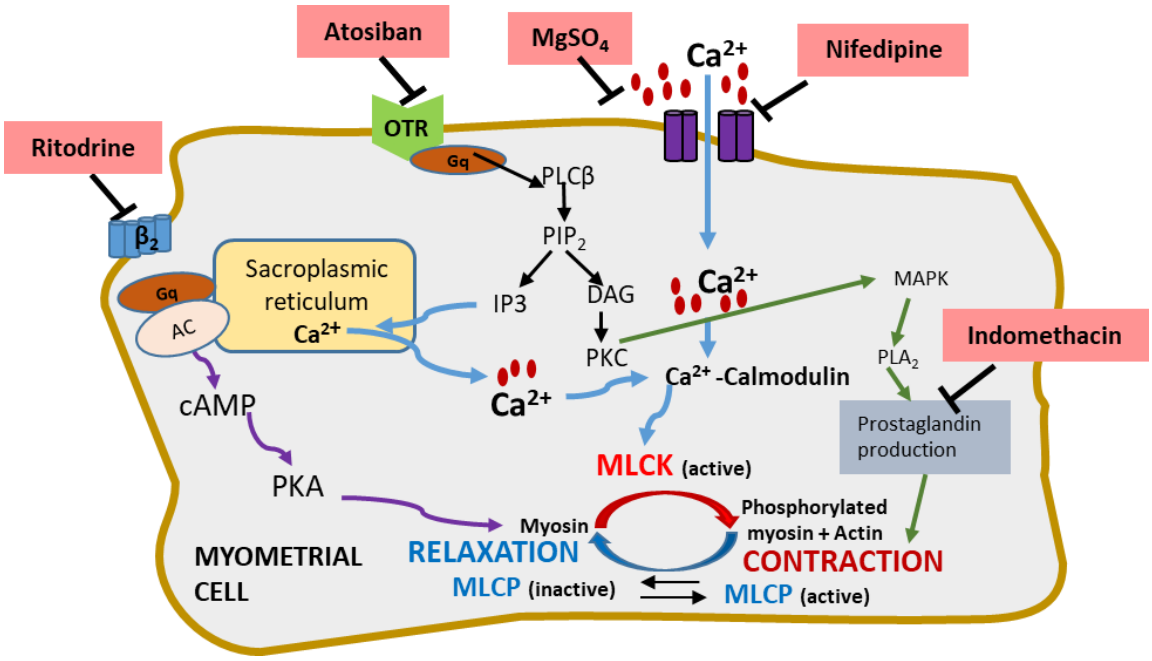


Figure 4. 1: Schematic showing the mechanism of action by different stimulants and relaxants on myometrial contractions

Ritodrine is a non-selective β–AR agonist whose tocolytic effect is mediated through the cAMP pathway. It acts by binding to β-AR, leading to increased levels of the cyclic AMP which activates protein kinase. Indomethacin, a prostaglandin-synthase inhibitor act via this pathway of inhibiting the contractility stimulator: prostaglandin. They inhibit cyclooxygenase COX-1 and COX-2, the relevant enzymes for the conversion of arachidonic acid to prostaglandins, leading to the reduced synthesis of prostaglandins thereby leading to reduced uterine contractions. Nifedipine, a calcium channel blocker acts by blocking the influx of Ca through the cell membrane and prevents the spread of action of action potential required for coordinated contractions of the myometrium.

The efficacy of tocolysis in stopping preterm labour has been greatly questioned. These tocolytics stop labour for a maximum period of 48 hours, allowing time for the administration of corticosteroids and maternal transfer to care centres to improve neonatal outcomes (Witcher, 2002). Even though the survival rate of very preterm babies (<30 weeks) has improved over the years, there is still a prevalence of neurodevelopmental challenges in these children (Usman et al., 2017). The risk of cerebral palsy, which is the general term used to describe the different types of motor and posture disabilities, increases with reduced gestation.

In 1988, a study revealed that preterm babies born to women with preeclampsia (and administered magnesium) previously had fewer germinal matrix haemorrhages (Leviton et al., 1988). A more recent study (Rouse et al., 2009), which involved 2,241 pregnant women between 24 – 31 weeks of gestation were either given magnesium sulphate or placebo. Results showed that infants were free of moderate or severe cerebral palsy at 2 years. These findings and many more findings recently led the Royal College of Obstetricians and Gynaecologists (RCOG) and National Institute for Health and Care Excellence (NICE) to recommend that magnesium sulphate is given to women who are likely to deliver before 30 weeks of gestation (NICE, 2015) This means that magnesium sulphate (which has a calcium antagonism mechanism) is currently given for neuroprotection in very preterm cases in combination with a tocolytic (e.g. indomethacin, nifedipine or atosiban).

Currently, there is the absence of substantial evidence to affirm that any tocolytic is more effective than another; the choice of any tocolytic is mainly on its safety over others (de Heus et al., 2009). Since tocolytics act via different signalling mechanisms, it was therefore hypothesized that a combination of magnesium and another tocolytic agent could result in a greater inhibitory effect (additive or synergistic), especially with drugs that target different pathways. In addition, this could lead to a lesser dosage needed and potentially decrease the rate of adverse effects seen with tocolytics whilst maintaining efficacy.

The aims of this chapter, therefore, were, in term pregnant mouse myometrium:

(v) To determine the effect of indomethacin, atosiban, and nifedipine on spontaneous and oxytocin-induced contractions, and calculate their respective IC_{50} values;

(vi) To examine the effect of magnesium in combination with indomethacin compared to Mg alone;

(vii) To examine the effect of magnesium in combination with atosiban compared to magnesium alone;

(viii) To examine the effect of magnesium in combination with nifedipine on term pregnant mouse uterine contractility compared to Mg alone;

(ix) To determine which combination of tocolytic is most effective in reducing term pregnant mouse uterine contractions.

During parturition, myometrial contractility is affected by several factors including hormonal changes, hypoxia, pH changes, lactate and stretch (Wray, 2007). Alterations in pH play a major role in myometrial contractions and the effect of these changes have been widely studied in human and animal models but the outcome hasn't been consistent. During pregnancy, plasma pH has been shown to increase while normal labour is associated with acidemia (Cerri et al., 2000). This may be explained by the occlusion of blood vessels resulting from strong uterine contractions (Brinkman, 1990). Since labour is affected by pH changes and reduced pH_o may affect force, it is important to investigate how this change may affect tocolytic efficacy. Therefore, a further aim of this chapter was to determine:

(x) The effect of reduced extracellular pH (pH_o) on uterine contraction and combined tocolytic efficacy.

4.3 Methods

4.3.1 Tissue and force measurement

All uterine strips used in this chapter were obtained from term pregnant mouse. As described in chapter 2, uterine strips were cleaned, dissected and prepared for force measurement. Uterine strips measuring 1 x 2 x 10mm were dissected and tied using a surgical thread on both ends. They were then mounted between a fixed hook and a force transducer and continuously perfused with PSS at 37^{o} C, bubbled with 95% O_2 and 5% CO_2 at a flow rate of 4ml/min and contractile activity was seen almost immediately the tissue was mounted. A 5mN stretch was applied on the tissues at the start of each experiment.

This chapter consists of: investigating the concentration-dependent effect of indomethacin alone (n=6); investigating the effect of magnesium and indomethacin (n=13); investigating the concentration-dependent effect of atosiban alone (n=4); investigating the effect of magnesium and atosiban (n=11); investigating the effect of nifedipine alone (n=7); investigating the effect of magnesium and nifedipine (n=7) and investigating the effect of acidification on Mg and Atosiban (n=7).

4.3.2 Solutions

Solutions including PSS were made as described in chapter 2. Oxytocin was used at 0.5nM concentration for all experiment in this chapter. Atosiban was used at 500nM concentration, indomethacin was used at 3nM for spontaneous and 30nM for oxytocin-induced experiments while Nifedipine was used at 0.01nM for both spontaneous and oxytocin-induced experiments. Details on how these drugs were made are detailed in **Chapter 2**.

4.3.3 Experimental protocol

In all uterine strips, contractile activity was observed immediately or after a few minutes of perfusion with HEPES buffered PSS. The strips were allowed to contract spontaneously until stable contractions were obtained before application of any drugs.

Concentration-dependent Experiments

The first part of this study investigated the effect of individual tocolytics on uterine contractions. Concentration-dependent experiments were carried out to determine a suitable concentration of indomethacin, atosiban and nifedipine in combination with magnesium. These experiments were also performed to determine the suitability of the vehicle time controls and the length of experiments. For spontaneous contractions, myometrial strips were left to equilibrate for a period of 45 – 60 minutes or until stable contractions were achieved. For oxytocin-induced contractions, once stable contractions were achieved as previously described, contractions were then stimulated with 0.5nM oxytocin. Increasing concentrations of each drug was then added at 15 minute interval. Indomethacin was examined at concentrations of 1, 3, 10, 30, 100, and in some cases 300µM, atosiban was examined at concentrations of 10nM, 30nM, 100nM, 300nM, 1µM, and 3 µM, and nifedipine was examined at concentrations of 3pM and 10pM (in some cases), 30pM, 100pM, 300pM, 1nM and 3nM. For oxytocin-induced experiments, all drugs were applied in the continuous presence of 0.5nM oxytocin. For all indomethacin experiments, the concentration of DMSO did not exceed 0.3% (v/v).

Combination Experiments

For spontaneous contractions, after recording control activity, the effect of the application of Mg and either of the drugs was examined. When combining Mg with indomethacin, Mg was applied in increasing concentrations (2-10mM) in the presence of indomethacin, at its predetermined IC_{50} value. Each concentration of Mg was applied for a 15-minute period as explained in Chapter 2. With Mg and nifedipine, the IC_{50} of Mg in spontaneous contractions was chosen (4mM) in combination with 0.01nM nifedipine. This protocol is different from indomethacin due to nifedipine's effect on contractions (details explained in the results section– 3.4.4). With Mg and atosiban, no experiments were carried out on spontaneous contractions.

In oxytocin-induced contractions, after stable spontaneous contractions were obtained, oxytocin (0.5nM) was applied. A stable control period with oxytocin was then recorded before application of reagents. When combining Mg with atosiban, a single concentration of atosiban was used (500nM) with increasing concentration of magnesium (2-12mM). Each concentration of Mg was applied for a 15-minute period. This protocol was the same for Mg and indomethacin (30µM). With Mg and nifedipine, the approximate IC_{50} value of Mg in oxytocin-induced contractions was used (10mM) in combination with 0.01nM nifedipine. Control experiments for all manoeuvres were carried out simultaneously. Traces are shown in Chapter 2.

The Effect of extracellular acidification

After obtaining stable spontaneous contractions, the tissue was stimulated with 0.5nM oxytocin and allowed to equilibrate. Once the contractions had contractions reached steady state, the combination Mg + atosiban pH 7.4 was added to the tissue for 15 minutes followed by Mg + atosiban pH 6.9 for another 15 minutes. The amplitude, frequency, duration and AUC of contractions were then calculated.

4.3.4 Data analysis and statistics

Data were analysed using Origin Pro 2015 and n is the number of myometrial tissue strips each from a different animal. Amplitude and duration of each contraction were measured during the 10 minutes of solution application and the mean calculated. Frequency was measured as the number of contractions within the 10-minute period. If there were no contractions during Mg application, amplitude and frequency of contractions were recorded as '0' and excluded from duration analysis. The area under the curve (AUC) was also measured using the last 10 minutes of solution application or control period. All data are presented as % of control period (100%). Values given are mean ± SEM unless stated otherwise. Significance was taken as $P<0.05$.

Concentration-response curves for AUC were fitted to the logistic equation with the use of non-linear regression. The concentration at which there was a 50% reduction in force integral (AUC) was calculated. The log IC_{50} values were calculated and

compared using the extra sum of squares F test or ANOVA followed by Bonferroni post-hoc analysis. All statistical analysis was carried out using GraphPad Prism 6.0.

Summary of statistical analyses performed are:

1) Tables 4.1 to 4.5- ANOVA with Bonferroni post hoc correction
2) Comparison of IC_{50} values for the effect of indomethacin alone on spontaneous and oxytocin-induced contractions – F-test
3) Comparison of IC_{50} values for spontaneous vs oxytocin in Mg + indomethacin combination – F-test
4) Comparison of IC_{50} values for Mg alone and Mg + indomethacin, Mg alone and Mg + atosiban - ANOVA with Bonferroni post hoc correction
5) Comparison of IC_{50} values between the combinations (Figure 4.10) – ANOVA with Bonferroni post hoc correction

4.4 Results

4.4.1 The Effect of Indomethacin on uterine contractions of term pregnant mouse

Concentration-response experiments were carried out to establish the concentration at which indomethacin caused a 50% reduction (IC_{50}) in spontaneous (n=7) and oxytocin-induced (n=6) contractions. In both spontaneous and oxytocin-induced experiments, indomethacin produced a concentration-dependent inhibitory effect on the force, frequency and AUC of contractions in all uterine strips examined. Representative isometric traces demonstrating the effect of indomethacin and vehicle time control (DMSO) on contractions are shown in Figure 4.2A-D. Mean data were used to plot bar charts and concentration-response curves representing the effect of indomethacin on force, frequency, duration and AUC on spontaneous (Figure 4.3A-D) and oxytocin-induced contractions (Figure 4.4 A-D). With the use of DMSO, there was no significant effect on spontaneous and oxytocin-induced contractions.

The IC_{50} values for AUC of spontaneous and oxytocin-induced contractions were 3.6 µM and 30.2 µM respectively. Statistical comparison of the IC_{50} values showed a significant difference between both groups ($P<0.001$). The concentration-response curves for the effect of indomethacin on oxytocin's activity when plotted against spontaneous activity, was shifted to the right (Figure 4.4E), indicating a higher IC_{50} value. For subsequent experiments, the approximated IC_{50} values of AUC were then used in combination with Mg (3µM and 30 µM for spontaneous and oxytocin-induced experiments respectively).

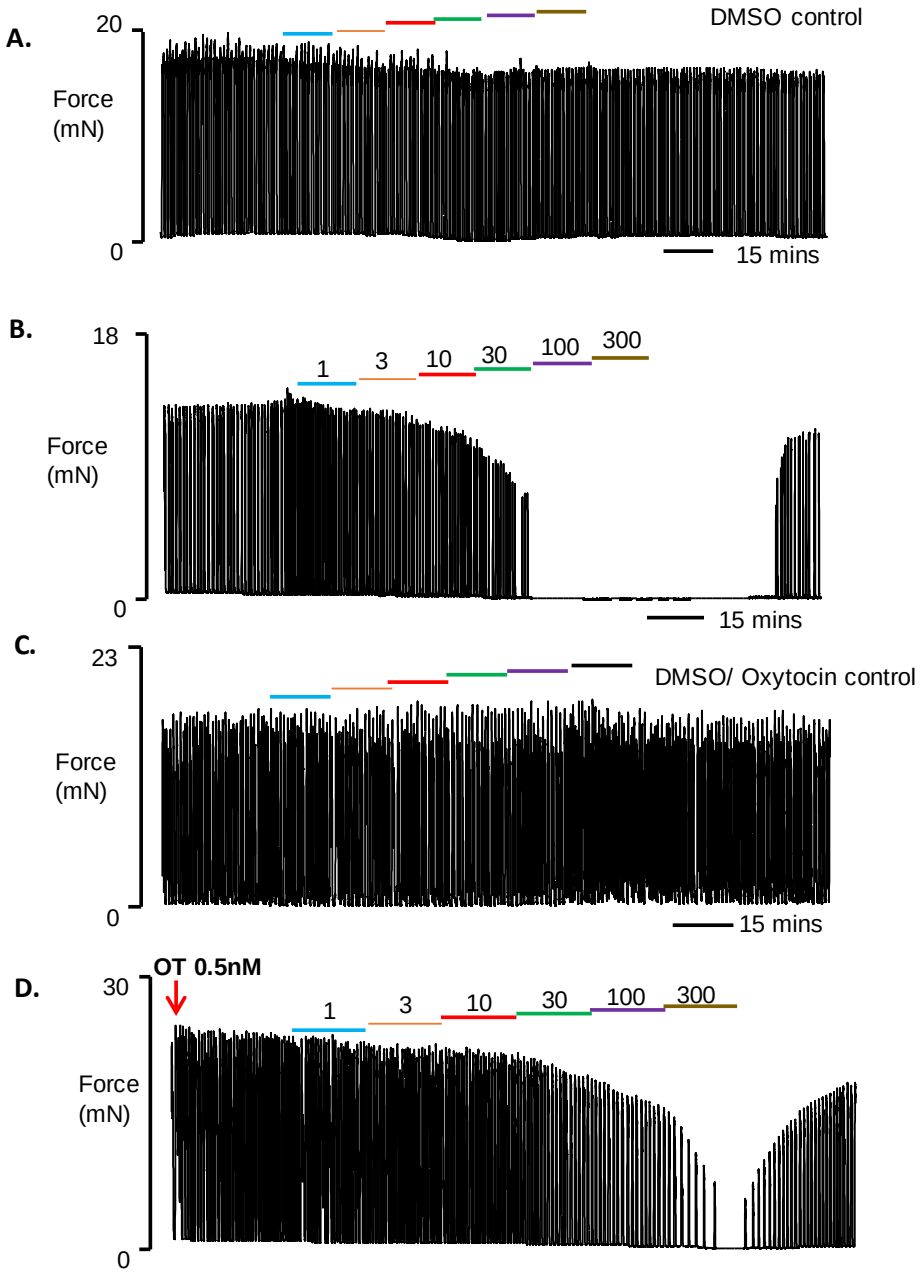


Figure 4. 2: The effect of Indomethacin on term mouse uterine contractions.

Representative isometric traces showing **A,** the effect of the vehicle control (DMSO) on spontaneous contractions, **B,** the effect of increasing concentration of indomethacin on spontaneous contractions, **C**, the effect of DMSO on oxytocin-induced contractions, and **D**, the effect of increasing concentration of indomethacin on oxytocin-induced contractions. The short coloured bars indicate the 15 minute period where increasing concentrations of DMSO or indomethacin were added. The blue (0.001% or 1µM) orange (0.003% or 3µM) red (0.01% or 10µM), green (0.03% or 30µM), purple (0.1% or 100µM) and brown (0.3% or 300µM) of DMSO or indomethacin respectively.

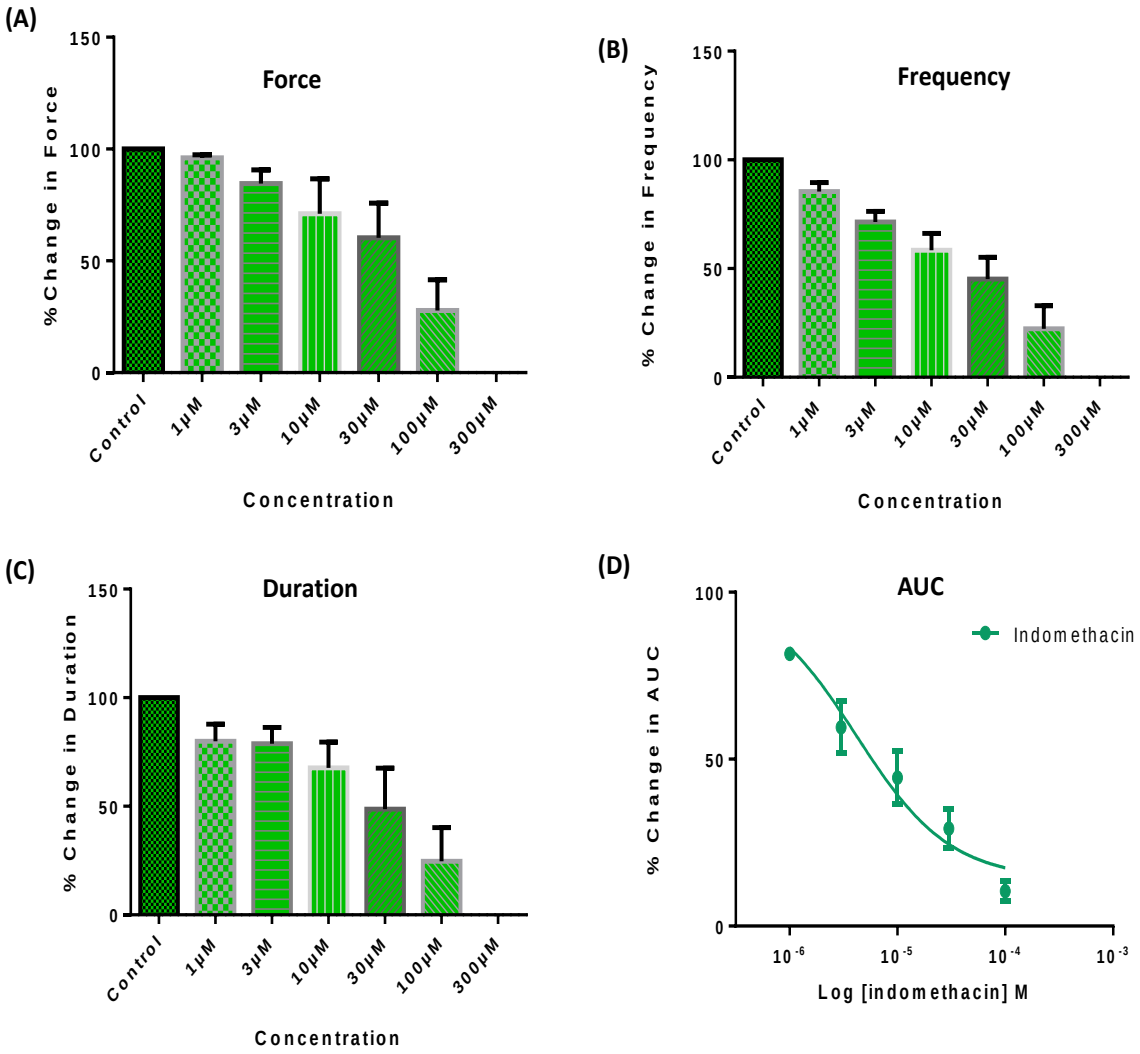


Figure 4. 3: Concentration dependency of Indomethacin on spontaneously contracting mouse myometrium

Average mean data ± SEM showing the concentration-dependent effect of Indomethacin on **A,** force, **B**, frequency, **C**, duration and **D,** AUC of spontaneously contracting strips.

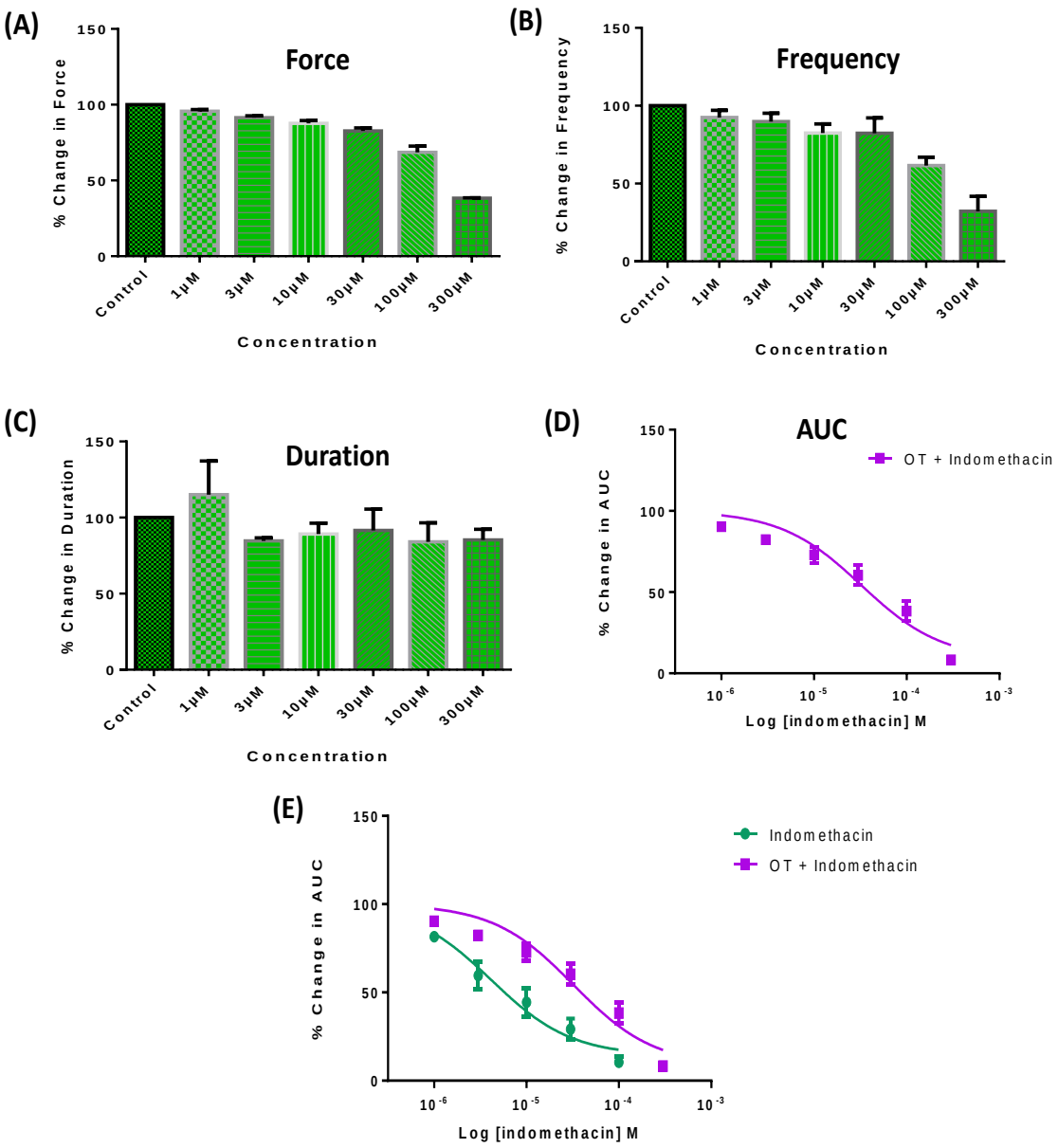


Figure 4. 4: Concentration dependency of Indomethacin on oxytocin-induced contracting term mouse uterus

Average mean data ± SEM showing the concentration-dependent effect of Indomethacin on (**A),** force, (**B**), frequency, (**C**), duration and (**D),** AUC of oxytocin-induced contractions. Figure **E** shows concentration-dependent curves for the effect of indomethacin on AUC of both spontaneous (green) and oxytocin-induced contractions (purple). A significant difference in activity was found using ANOVA with Bonferroni post hoc. *denotes $p<0.05$, ** $p<0.01$, ***$p<0.0001$.

4.4.2 The Effect of Mg + Indomethacin on uterine contractions of term pregnant mouse.

The inhibitory effect of Mg plus Indomethacin on spontaneous contractions was examined. Increasing concentration of Mg (2-10mM) were applied to spontaneously contracting myometrial strips for 15 minutes each in the presence of a predetermined single concentration of indomethacin (3µM) (Figure 4.5) and its effect on contractility was determined (Figure 4.6). Magnesium + Indomethacin on in spontaneously contracting myometrium, caused a concentration-dependent reduction of force amplitude, frequency and AUC of contraction in all samples examined (n=8). There was a significant decrease in the contractile parameters measured- force amplitude, duration, frequency and AUC of contractions when compared to the control period (see Table 4.1, ANOVA with Bonferroni post hoc analysis). At 2mM and higher, there was a significant decrease in the force of contractions. At 10mM Mg concentration, the force was reduced to 17.2% ± 11.3 (relative to control, 100%), $P<0.0001$. At 4mM or higher, a sudden and marked drop or abolition of contraction force was observed I n 7 of 8 tissues. There was also a marked decrease in contraction frequency with increased Mg concentrations which was significant from the first concentration (2mM) onwards. At 10mM Mg, the frequency of contractions was reduced to 10.9% ± 6.7 ($P<0.0001$). The duration of contractions at 10mM was reduced to 26.0% ± 12.7 ($P<0.05$) compared to control. The AUC however, which integrates all three parameters showed a significant concentration-dependent decrease. At 10mM Mg, the AUC was reduced to 4.6% ± 3.1 compared to control (n=8).

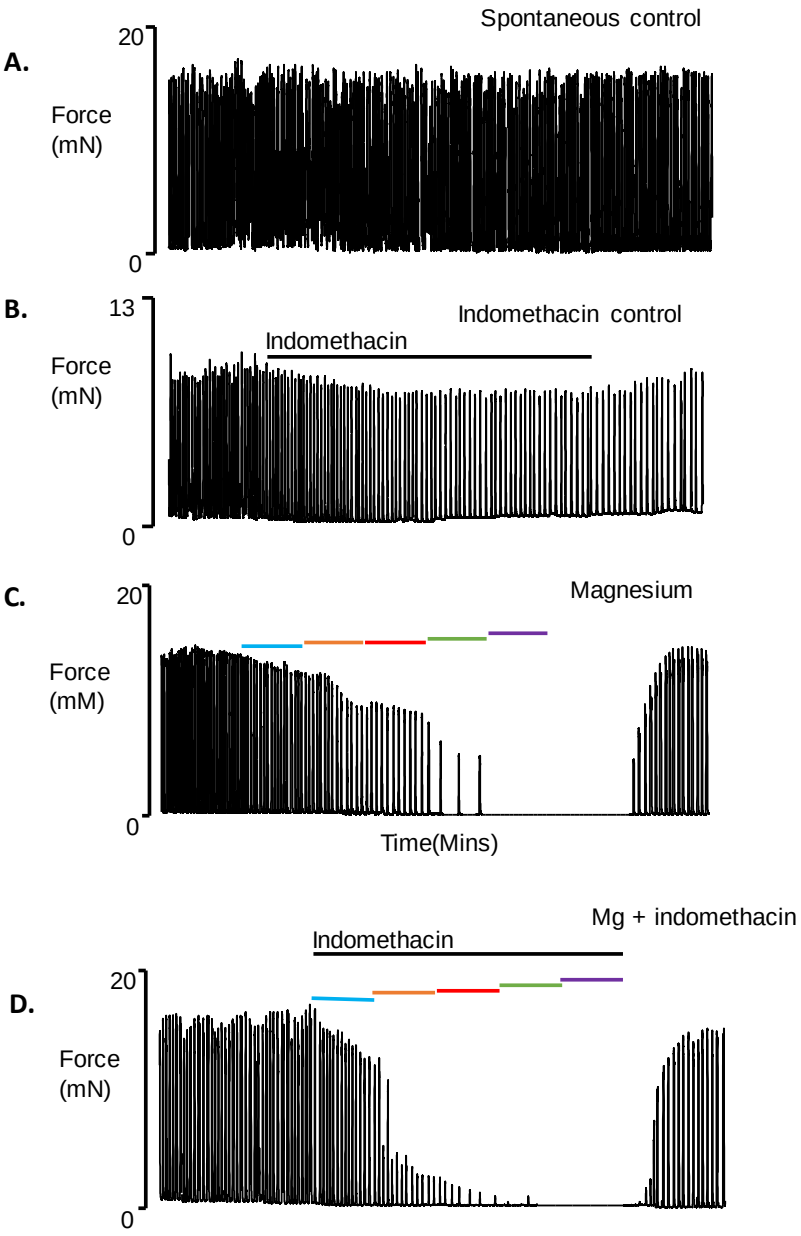


Figure 4. 5: The effect of $MgSO_4$ and Indomethacin on spontaneously contracting mouse myometrium.

Representative isometric traces showing **A,** spontaneous time control **B,** indomethacin (3uM) alone, **C**, the effect of increasing concentration of magnesium sulphate on spontaneous contractions, and **D**, the effect of magnesium sulphate in combination with indomethacin on spontaneous contractions. Magnesium concentration dependently reduces the contraction and this inhibitory effect is enhanced when combined with indomethacin. The short coloured bars indicate the 15 minute period where magnesium was added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), and 10mM (purple).

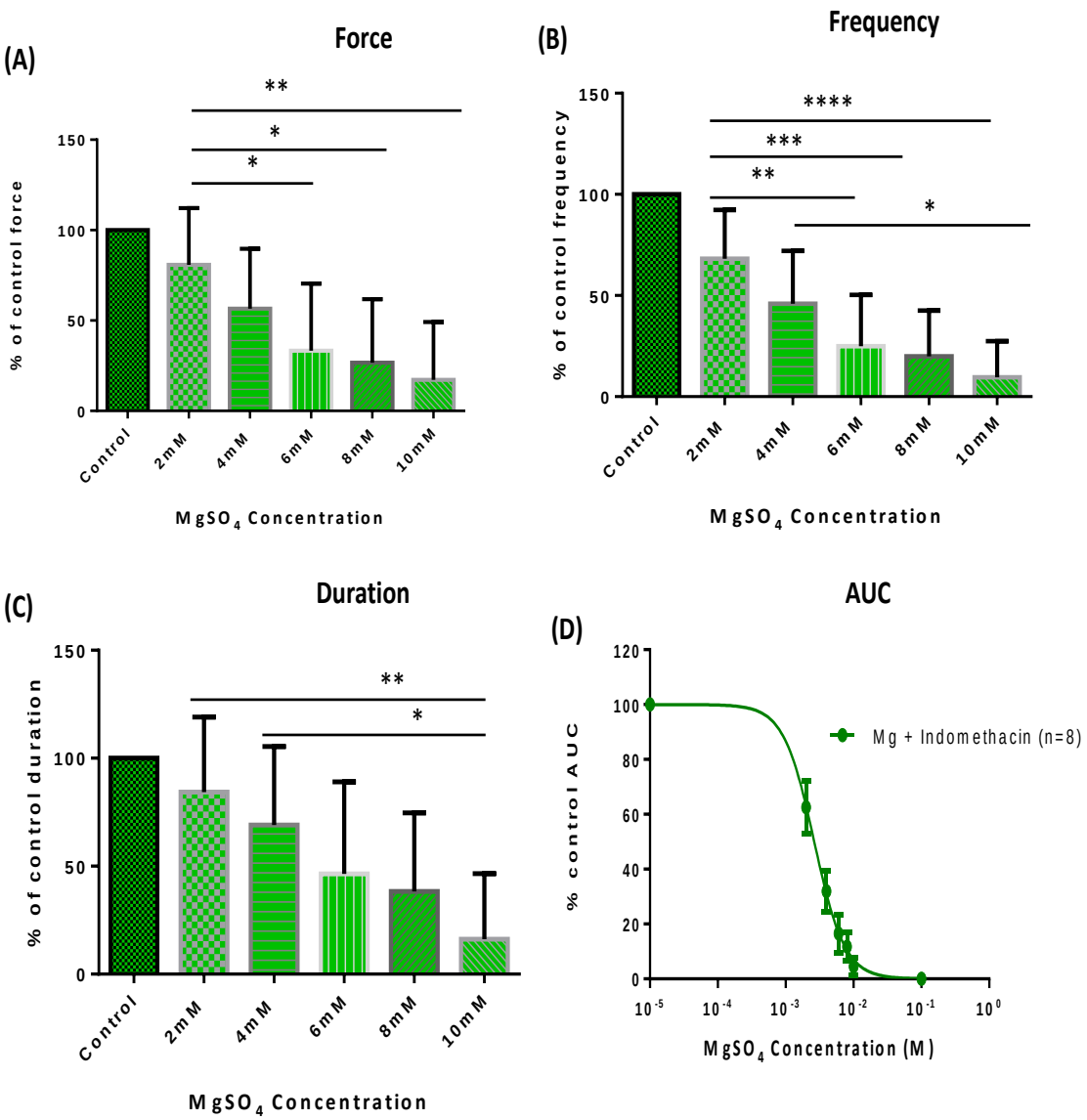


Figure 4. 6: Concentration dependency of $MgSO_4$ + Indomethacin on spontaneously contracting mouse myometrium

Mean data ± SEM showing the concentration-dependent effect of $MgSO_4$+Indomethacin in **A,** force, **B**, frequency, **C**, duration and **D** AUC of spontaneously contracting strips. A significant difference in activity was found using ANOVA with Bonferroni post hoc. *denotes p<0.05, **p<0.01, ***p<0.001, **** p<0.0001

(A)

	$MgSO_4$	Mg+ Indomethacin	Significance level
2mM	91.8 ± 2.7	80.8 ± 11.1 **	ns
4mM	61.8 ± 10.9 *	56.6 ± 11.7 ***	ns
6mM	56.9 ± 11.5 *	33.4 ± 13.1 ****	ns
8mM	38.3 ± 9.9 ***	26.7 ± 12.4 ****	ns
10mM	19.6 ± 9.8 ****	17.2 ± 11.3 ****	ns

(B)

	$MgSO_4$	Mg + Indomethacin	Significance level
2mM	72.5 ± 6.7*	76.0 ± 3.7 **	ns
4mM	43.6 ± 8.2****	52.5 ± 7.1 ***	ns
6mM	35.5 ± 7.8****	28.5 ± 8.9 ****	ns
8mM	20.5 ± 7.1****	22.8 ± 8.1 ****	ns
10mM	9.6 ± 5.0****	10.9 ± 6.7 ****	ns

(C)

	$MgSO_4$	Mg + Indomethacin	Significance level
2mM	110 ± 5.3	63.6 ± 13.6	ns
4mM	76.8 ± 17.3	49.2 ± 12.1	ns
6mM	74.6 ± 21.5	31.7 ± 11.6	ns
8mM	64.4 ± 25.1	26.4 ± 10.2 *	ns
10mM	44.3 ± 23.3	26.0 ± 12.7 *	ns

(D)

	$MgSO_4$	Mg + Indomethacin	Significance level
2mM	71.3 ± 6.4 **	62.5 ± 9.5 **	ns
4mM	43.3 ± 6.7***	32.0 ± 7.6 ****	ns
6mM	33.1 ± 6.5***	16.5 ± 7.1 ****	ns
8mM	18.0 ± 5.4***	11.8 ± 5.1 ****	ns
10mM	6.1 ± 3.2 ***	4.6 ± 3.1 ****	ns

Table 4. 1: A comparison of the effect of Magnesium alone and Mg + indomethacin on spontaneous contractions.

Mean data ± SEM showing concentration dependent decrease in (A) amplitude force (B) frequency (C) duration and (D) AUC in spontaneously contracting term pregnant mouse uterine strips. Significance level was determined by ANOVA Bonferroni post hoc analysis. * indicates P<0.05, **P<0.01, P<0.001, P<0.0001

A concentration response curve was plotted to show the AUC of spontaneous contractions and the IC_{50} (the concentration at which contractile activity is reduced by 50%) value for Mg + Indomethacin was determined as 2.6 ± 0.4 mM compared with 3.08 ± 0.16 mM for Mg alone.

Different contractile properties could be affected under agonist stimulated conditions; therefore, the effect of Mg + Indomethacin on oxytocin-induced contractions was also examined. 0.5nM oxytocin was applied to stable spontaneous contractions.

Increasing concentrations of Mg (2-12mM) were added to oxytocin-induced contracting tissues (Figure 4.7). As observed in spontaneous contractions, Mg + Indomethacin produced a concentration-dependent decrease in oxytocin-induced contractions in all tissues, however. In contrast to spontaneous contraction, this was not sudden in onset, but a gradual decline in activity. Myometrium from a total of 8 mice was used in investigating the effect of Mg + Indomethacin on oxytocin-induced contractions and its effect on contractile parameters were calculated (see Table 4.2).

At 2mM and higher, there was a significant decrease in the force of contractions. At 12mM Mg concentration, the force was reduced to 70.6% ± 0.2 ($P<0.001$). There was also a marked decrease in contraction frequency with increased Mg concentrations which was significant from 4mM onwards. At 12mM Mg, the frequency of contractions was reduced to 69.6% ± 0.3 (relative to control, 100%), $p<0.0001$. The duration of contractions at 12mM was reduced to 89.7% ± 10.7 and wasn't statistically significant. The AUC however, which integrates all three parameters showed a significant concentration-dependent decrease.

At 12mM Mg, it was reduced to 1.4% ± 1.0 compared to control (n=8). 70.6% ± 3.1 ($P<0.001$). The AUC values were used to plot a concentration-response curve as a percentage of control activity. The changes in contractile parameters are represented in the bar charts and concentration-response curve shown in Figure 4.8. From this concentration-response curve, the IC_{50} for Mg + Indomethacin in the

presence of oxytocin was calculated and determined as 12.3 ± 1.5 mM compared to 9.8 ± 0.2 mM for Mg alone.

To determine the concentration at which contractions are completely abolished, a separate experimental manoeuvre was carried out. A concentration of 10mM and 20mM Mg was applied in combination with 30µM Indomethacin. At 20mM, there was the complete abolition of contractions (Figure 4.7E). Its effect on different contractile parameters is indicated in the bar charts and concentration-response curve in Figure 4.8.

In comparison between spontaneous and oxytocin-induced contractions, it was observed that a greater effect of the combination was seen in spontaneous contractions (Figure 4.9). When the concentration-response showing the effect of Mg + Indomethacin on oxytocin is plotted against that of spontaneous activity, it is observed that the curve is significantly shifted to the right demonstrating a higher IC_{50} value ($P<0.0001$).

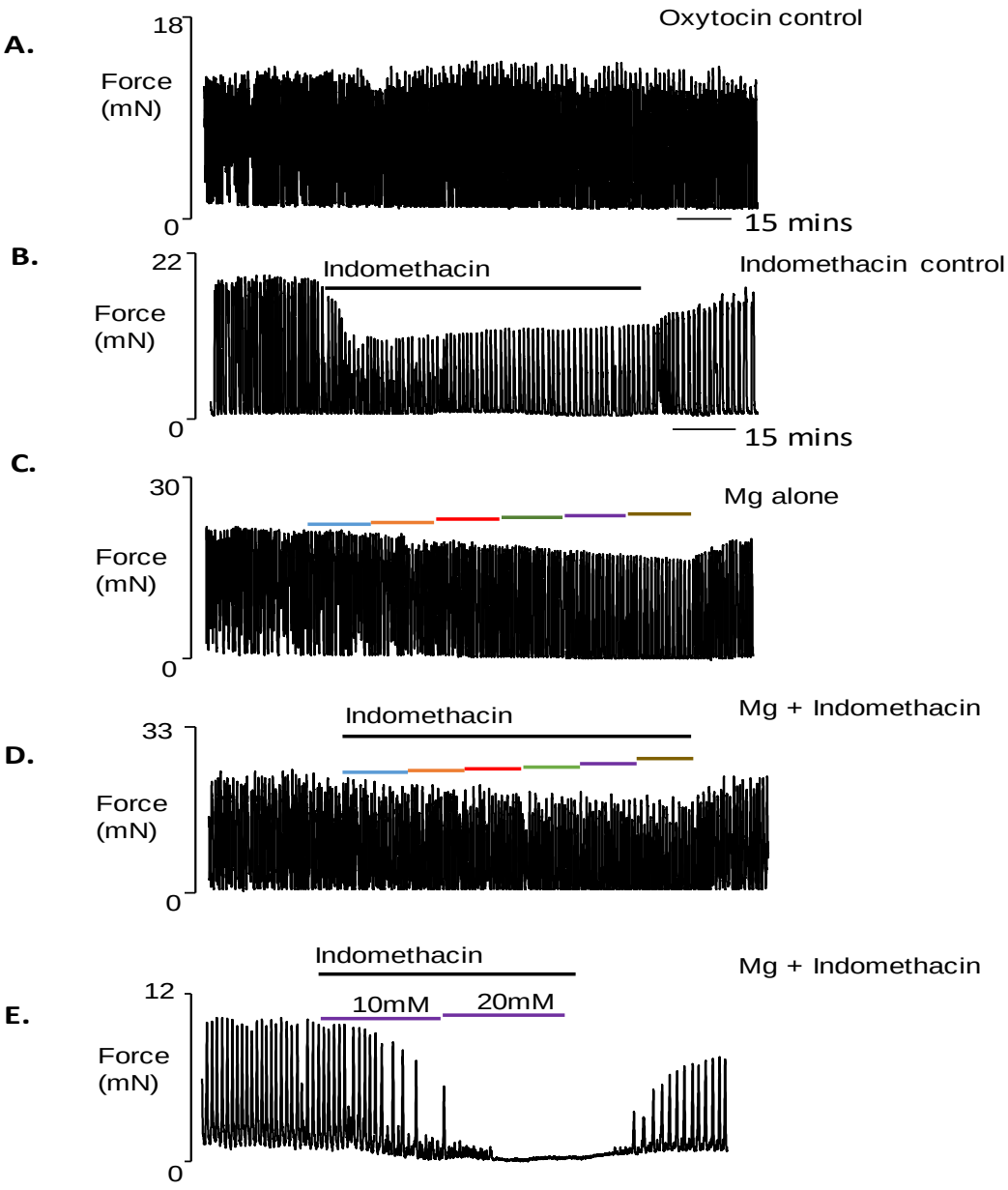


Figure 4. 7: The effect of $MgSO_4$ and Indomethacin on oxytocin-induced contractions of mouse myometrium

Representative traces showing **A**, oxytocin time control, **B**, indomethacin (30µM) time control, **C**, the effect of increasing concentration of magnesium sulphate in oxytocin-induced contractions, **D**, the effect of magnesium sulphate and indomethacin on oxytocin-induced contractions and **E**, the effect of magnesium sulphate (10mM and 20mM) and 30µM indomethacin in all traces were in the presence of oxytocin. The short coloured bars indicate the 15-minute period where different concentrations of magnesium were added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), 10mM (purple) and 12mM (brown).

(A)

	$MgSO_4$	$MgSO_4$+ Indomethacin	Significance level
2mM	98.8 ± 1.0	93.7% ± 1.5 **	ns
4mM	95.2 ± 1.2	85.9% ± 1.7 ***	ns
6mM	90.6 ± 1.7	81.0% ± 2.2 ***	ns
8mM	84.0 ± 2.0*	77.1% ± 2.4 ***	ns
10mM	71.3 ± 7.4****	73.6% ± 2.5 ***	ns
12mM	71.0 ± 4.9****	70.6% ± 0.2 ***	ns

(B)

	$MgSO_4$	$MgSO_4$+ Indomethacin	Significance level
2mM	93.9 ± 2.3	96.0% ± 2.1	ns
4mM	88.3 ± 3.2	92.9% ± 1.8 *	ns
6mM	77.2 ± 3.2****	87.8% ± 1.9 **	ns
8mM	65.7 ± 3.4****	82.4% ± 1.4 ****	ns
10mM	57.9 ± 3.3****	74.6% ± 2.9 ***	***
12mM	58.0 ± 3.9****	69.6% ± 0.3 ****	***

(C)

	$MgSO_4$	$MgSO_4$+ Indomethacin	Significance level
2mM	101.4 ± 2.8	81.2% ± 7.8	ns
4mM	109.7 ± 7.0	88.9% ± 6.8	ns
6mM	106 ± 5.4	79.6% ± 6.9	ns
8mM	109.3 ± 6.3	86.0% ± 8.9	ns
10mM	100.9 ± 15.0	83.6% ± 9.8	ns
12mM	112.2 ± 10.7	89.7% ± 10.7	ns

(D)

	$MgSO_4$	$MgSO_4$+ Indomethacin	Significance level
2mM	93.8 ± 1.2	93.7% ± 1.5	ns
4mM	83.3 ± 1.5	85.9% ± 1.7	ns
6mM	73.6 ± 2.6	81.0% ± 2.2	ns
8mM	60.9 ± 3.5	77.1% ± 2.4	ns
10mM	50.5 ± 3.9	73.6% ± 2.5	ns
12mM	41.3 ± 6.7	70.6% ± 3.1	**

Table 4. 2: A comparison of the effect of Magnesium alone and Mg + indomethacin on oxytocin-induced contractions.

Mean data ± SEM showing concentration dependent decrease in (A) amplitude force (B) frequency (C) duration and (D) AUC in oxytocin-induced contracting term pregnant mouse uterine strips. Significance levels were determined by ANOVA Bonferroni post hoc analysis. * indicates P<0.05, **P<0.01, P<0.001, P<0.0001

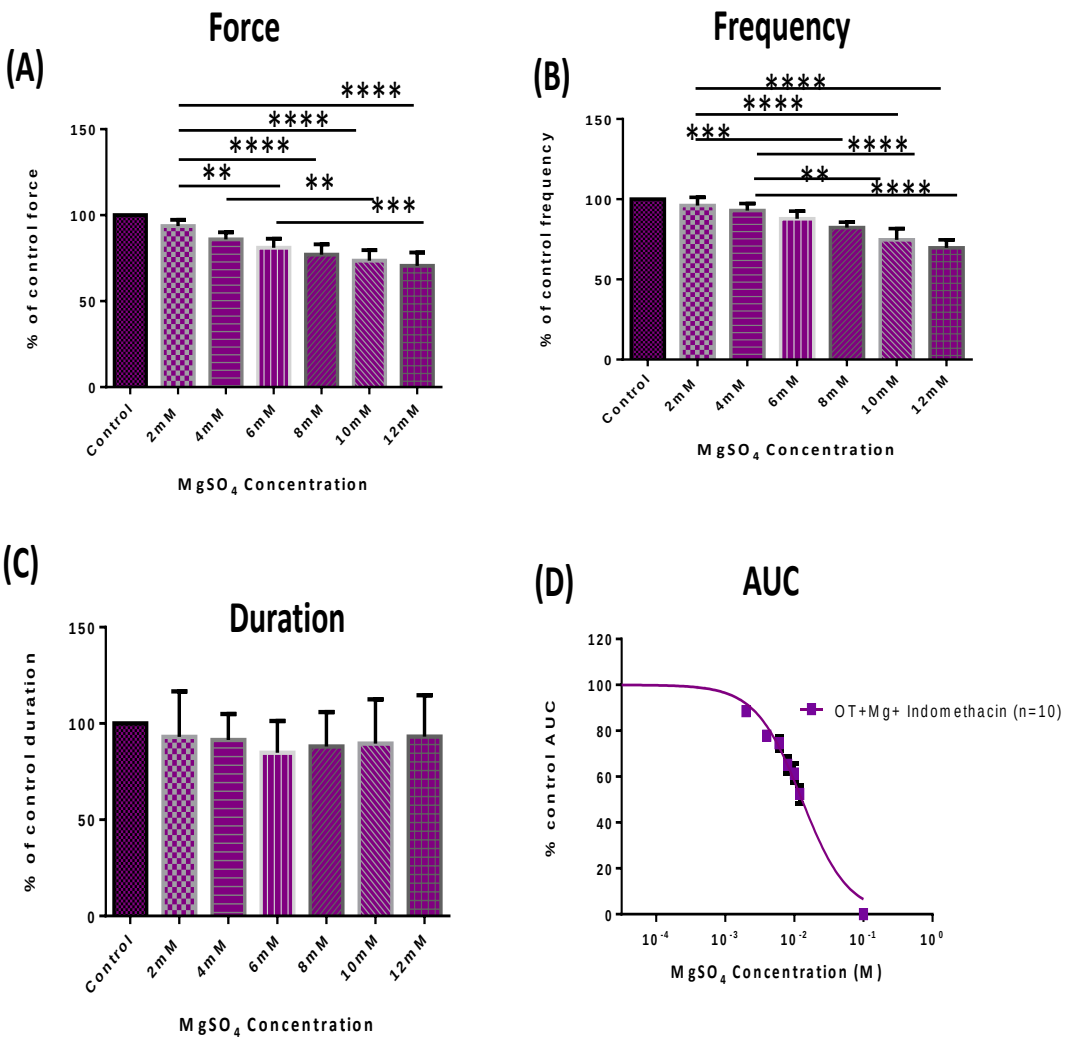


Figure 4. 8: Concentration dependency of $MgSO_4$ + Indomethacin on oxytocin-induced contracting mouse myometrium

Mean data ± SEM showing the concentration-dependent effect of $MgSO_4$+ Indomethacin in (A) force (B) frequency (C) duration and (D) AUC of oxytocin-induced contracting strips. A significant difference in activity was found using ANOVA with Bonferroni post hoc test. **denotes p<0.01, ****p<0.0001

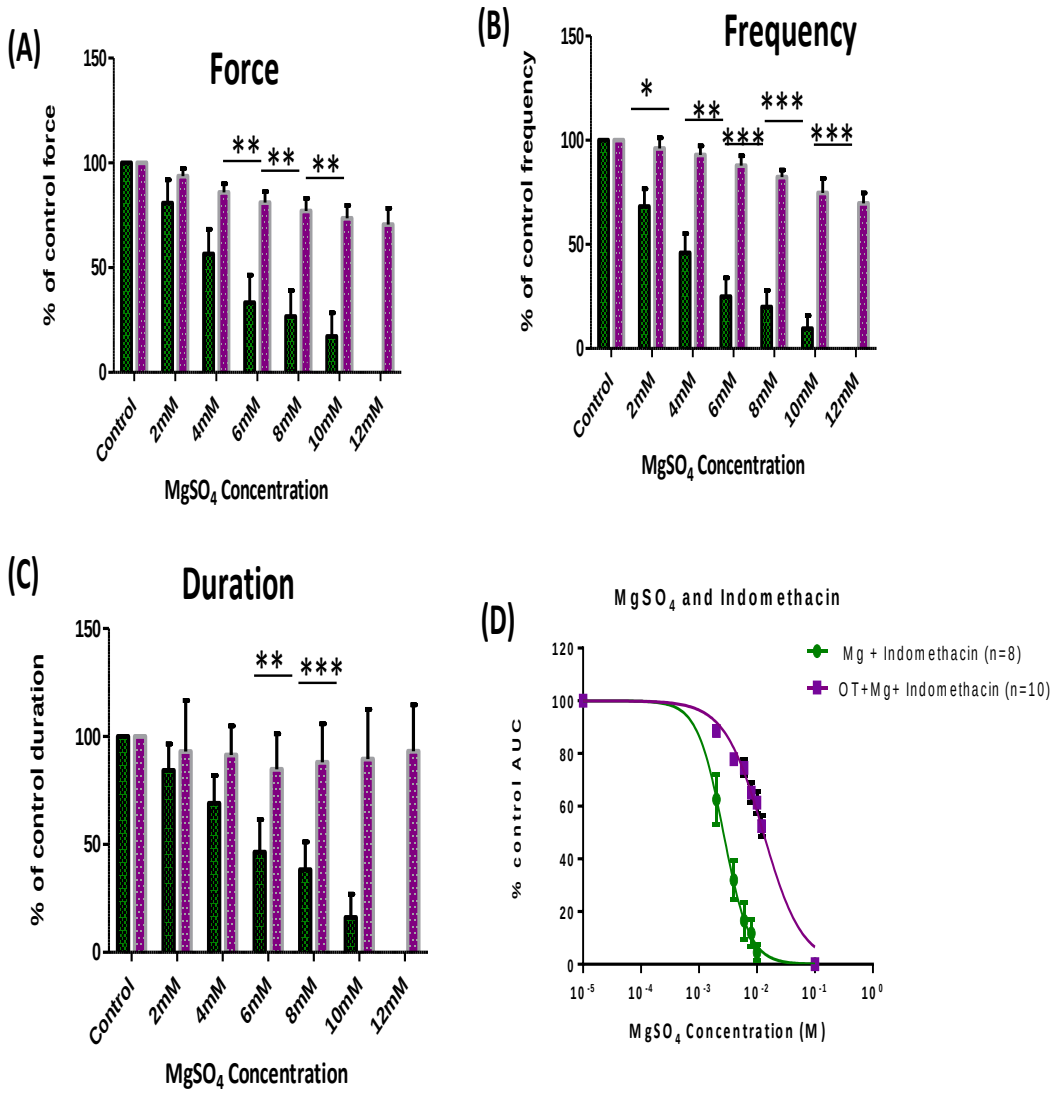


Figure 4. 9: A comparison of $MgSO_4$ + Indomethacin in spontaneous and oxytocin-induced contractions

Mean data ± SEM showing the dose-dependent effect of $MgSO_4$+Indomethacin in (A) force (B) frequency (C) duration and (D) AUC of spontaneously (green) and oxytocin-induced (purple) contracting strips. A significant difference in activity was found using ANOVA with Bonferroni post hoc. *denotes $p<0.05$, ** $p<0.01$, ***$p<0.0001$

4.4.3 The Effect of Atosiban on uterine contraction of term pregnant mouse.

Increasing concentrations of atosiban (10nM -3µM) was applied to oxytocin-induced contractions. Representative isometric traces demonstrating the effect of increasing concentration of atosiban and time control (oxytocin alone) are shown in Figure 4.10A-B. Data showing atosiban's effect on force, frequency and duration are represented on bar charts (Figure 4.11A-C), while its effect on AUC is represented in a concentration-response curve (Figure 4.11D).

The force of contractions was significantly reduced at 300nM and higher. The frequency was significantly reduced from 30nM and higher. Atosiban had no significant effect on the duration of contractions. The AUC values were used to plot a concentration response curve and the IC_{50} value for atosiban was determined as 376 nM ± 20.1. The IC_{50} (approximated) value used for subsequent combination experiment was 500nM.

4.4.4 The Effect of Mg plus Atosiban on uterine contractions of term pregnant mouse.

The effect of Mg plus atosiban was not determined in spontaneous contractions as our group has previously shown that atosiban had little or no effect on spontaneous contractions (Arrowsmith et al., 2015), therefore only oxytocin-induced contractions were investigated. Figure 4.12 shows typical recording of myometrial contractions stimulated with 0.5nM oxytocin (Figure 4.12A), inhibited by 0.5µM atosiban (Figure 4.12B), showing the effect of increasing concentrations of Mg (Figure 4.12C), and the inhibitory effect of Mg + atosiban (Figure 4.12D).

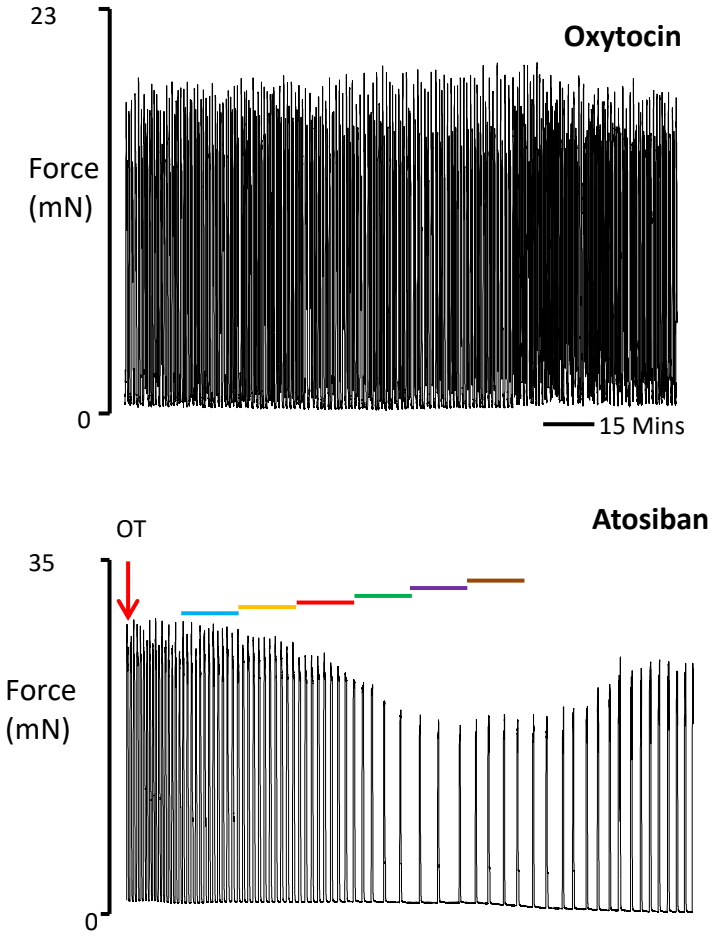


Figure 4. 11: The effect of Atosiban on oxytocin-induced contractions of term mouse myometrium

Isometric representative traces showing (**A)**, the effect of 0.5nM oxytocin on term pregnant mouse myometrium which continued contracting without loss of force and frequency over a long period of time and, (**B)**, the inhibitory effect of increasing concentrations of atosiban (10nM- 3µM) on oxytocin-induced contractions.
The short coloured bars indicate the 15-minute period where increasing concentrations of atosiban were added: 10nM (blue), 30nM (orange), 100nM (red), 300nM (green), 1µM (purple) and 3µM (brown).

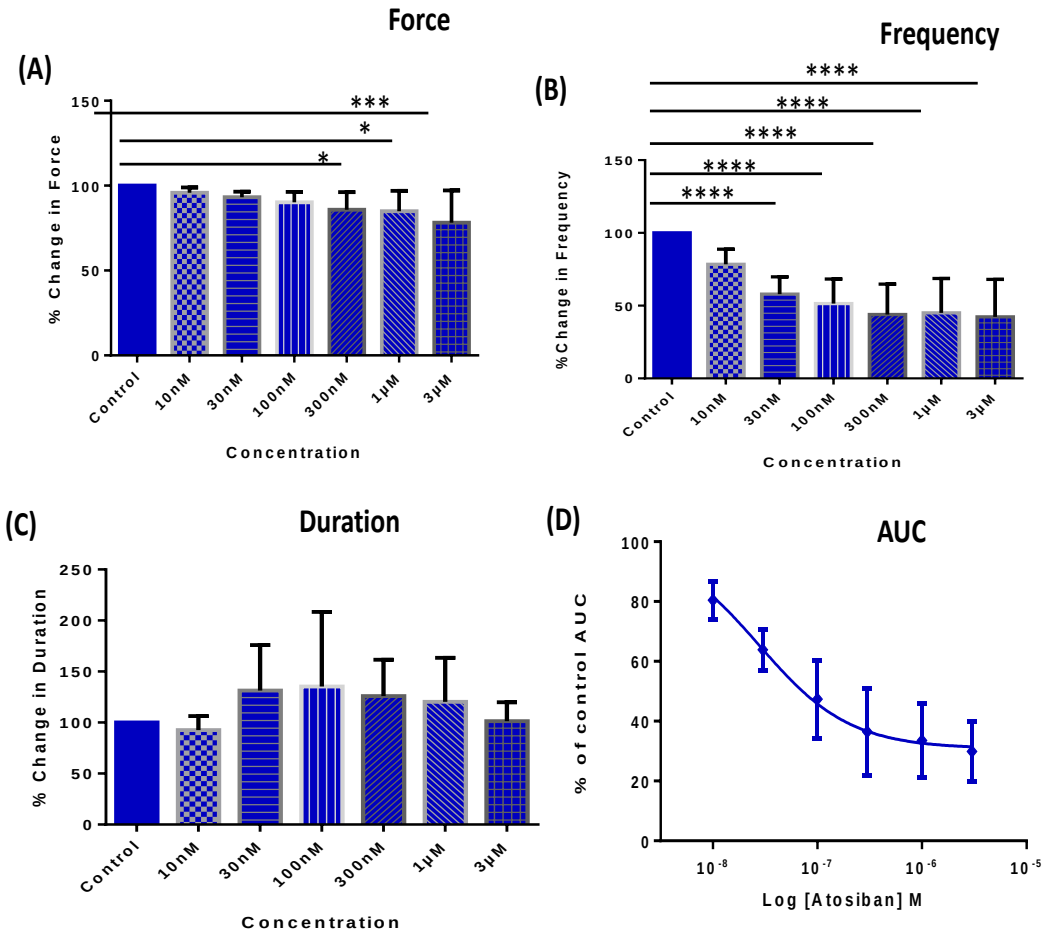


Figure 4. 12: Concentration dependency of Atosiban on oxytocin-induced contracting term mouse uterus

Average mean data ± standard error mean (SEM) showing the concentration-dependent effect of atosiban on (**A),** force, (**B)**, frequency, (**C)**, duration and (**D),** AUC of oxytocin-induced contractions. A significant difference in activity was found using ANOVA with Bonferroni post hoc. *denotes $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

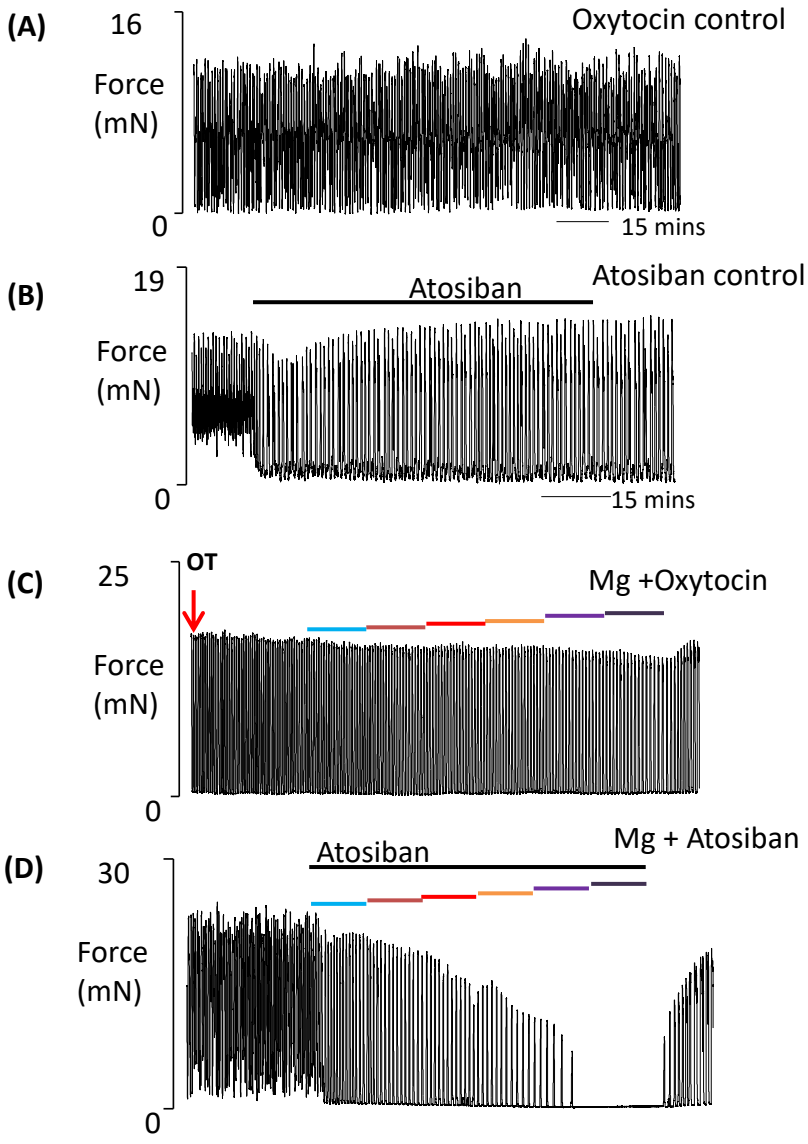


Figure 4. 13: The combined effect of Magnesium and atosiban on oxytocin-induced contractions

Representative isometric traces showing (A) oxytocin time control (B) atosiban time control (C) the effect of Magnesium on oxytocin-induced contractions and (D), the effect of Magnesium + Atosiban on oxytocin-induced contractions.
Magnesium alone slowly reduced the contractions, but when atosiban was added, contractions were inhibited and in almost all cases, completely abolished.
The short coloured bars indicate the 15-minute period where magnesium was added. 2mM (blue), 4mM (orange), 6mM (red), 8mM (green), 10mM (purple) and 12mM (brown).

In the presence of oxytocin, Mg + Atosiban caused a concentration-dependent decrease on force, frequency and AUC. At 2mM and higher, there was a significant decrease in the force of contractions (Table 4.3). At 12mM Mg concentration, the force was reduced to 9.7% ± 9.7 with of 6 of 8 samples completely abolished. There was also a marked decrease in contraction frequency with increased Mg concentrations which was significant from the first concentration (2mM) onwards. At 12mM Mg, the frequency of contractions was reduced to 2.3% ± 2.8 (relative to control, 100%), $p<0.001$. The duration of contractions at 12mM was reduced to 11.12% ± 10.4 compared to control. The AUC however, which integrates all three parameters showed a significant concentration-dependent decrease. At 12mM Mg, the AUC was reduced to 1.4% ± 1.0 compared to control (n=8).

The concentration-dependent reduction in oxytocin-induced contractions is represented in the bar charts (Figure 4.13) for force (Figure 4.13A), frequency (Figure 4.13B), and duration (Figure 4.14C). To determine the IC_{50} value, a concentration-response curve was plotted using the AUC values (Figure 4.13D) of Mg alone and in combination with atosiban. Mg + atosiban caused a leftward shift of the concentration-response curve compared with Mg alone. The IC_{50} values for Mg + Atosiban were 3.2 mM ± 0.04 compared with 7.4 mM ± 1.4 for Mg alone ($p<0.001$).

4.4.5 Comparison between Mg + Indomethacin and Mg + Atosiban

In oxytocin-induced contractions, the same experimental procedures were used to test the tocolytic efficacy of Mg + Atosiban and Mg + Indomethacin on pregnant mouse myometrium; therefore it was possible to make a direct comparison between these drug combinations.

(A)

	Mg	Mg + Atosiban	Significance level
2mM	96.6 ± 1.1	90.8 ± 2.8	ns
4mM	90.0 ± 3.1	86.9 ± 2.9	ns
6mM	83.9 ± 5.0	80.0 ± 4.9	ns
8mM	78.4 ± 5.7	70.3 ± 7.5	ns
10mM	65.7 ± 10.6	49.3 ± 11.9	*
12mM	55.6 ± 0.7	9.7 ± 9.7	***

(B)

	Mg	Mg + Atosiban	Significance level
2mM	79.0 ± 4.7	66.6% ± 7.0	ns
4mM	71.2 ± 5.8	52.3% ± 4.2	ns
6mM	65.3 ± 7.0	42.4% ± 4.0	*
8mM	46.8 ± 7.9	33.8% ± 4.3	ns
10mM	33.6 ± 10.1	17.1% ± 5.7	ns
12mM	21.4 ± 8.7	2.3% ± 2.3	ns

(C)

	Mg	Mg + Atosiban	Significance level
2mM	93.8 ± 1.2	95.6 ± 7.7	ns
4mM	83.3 ± 1.5	90.2 ± 9.0	ns
6mM	73.6 ± 2.6	82.9 ± 6.7	ns
8mM	60.9 ± 3.5	77.6 ± 7.8	*
10mM	50.5 ± 3.9	83.2 ± 10.2	ns
12mM	41.3 ± 6.7	11.2 ± 11.2	***

(D)

	Mg	Mg + Atosiban	Significance level
2mM	92.6 ± 9.1	61.6% ± 13.4	*
4mM	67.6 ± 6.2	46.5% ± 11.5	ns
6mM	55.2 ± 7.2	33.9% ± 10.7	*
8mM	43.4 ± 10.0	25.2% ± 9.6	*
10mM	35.4 ± 8.3	11.9% ± 2.2	**
12mM	30.2 ± 4.2	1.4% ± 1.0	**

Table 4. 3: A comparison of the effect of Mg alone and Mg + atosiban on oxytocin-induced contractions

Mean data ± SEM showing concentration dependent decrease in (A) amplitude force (B) frequency (C) duration and (D) AUC in oxytocin-induced contracting term pregnant mouse uterine strips. Significance levels were determined by ANOVA Bonferroni post hoc analysis. * indicates P<0.05, **P<0.01, P<0.001.

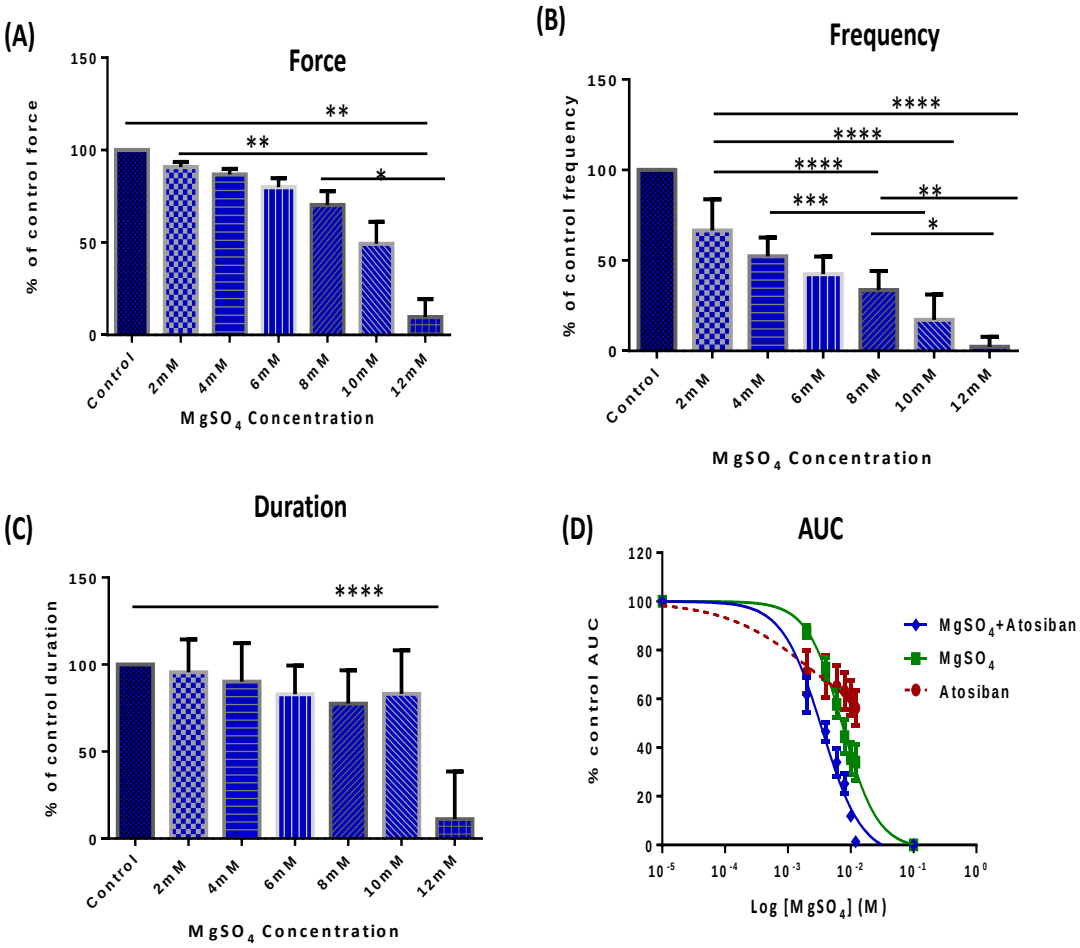


Figure 4. 14: Concentration dependency of $MgSO_4$ + Atosiban on oxytocin-induced contracting mouse myometrium

Bar charts showing the concentration-dependent effect of $MgSO_4$ + atosiban on force **(A),** frequency **(B)** and duration **(C)**. **D,** shows concentration response curves depicted for the effect of $MgSO_4$ alone (green) and when combined with atosiban (blue). The concentration response curve for atosiban time control (red) is also plotted. With the combination, the curve is significantly shifted to the left for AUC ($P<0.0001$). A significant difference in activity was found using ANOVA with Bonferroni post hoc. **denotes $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Data are presented as mean ± SEM.

The mean data for both drug combinations are indicated in Table 4.5 and these values were used to plot bar chart for force, frequency and duration (Figure 4.14A-D) while a concentration-response curve was plotted to reflect the changes in AUC (Figure 4.14D). The IC_{50} values for Mg + Atosiban and Mg + Indomethacin were 3.2 mM ± 0.04 and 12.2 mM ± 1.5 respectively. There was no significant difference between the drug combinations in force (Figure 4.14A), and duration (Figure 4.14C), from 2-10mM Mg, however, a significant difference was observed at 12mM Mg. The difference seen in frequency was significantly different at all concentrations of Mg (Figure 4.14B). The concentration-response of Mg + Indomethacin was significantly shifted to the right ($P<0.001$) indicating a higher IC_{50}.

4.4.6 The Effect of Nifedipine on Uterine Contractions of term pregnant mouse.

To determine the IC_{50} value of nifedipine on spontaneous and oxytocin-induced contractions, a similar protocol to indomethacin and atosiban was used. Increasing concentrations of nifedipine (3pM- 3nM) were used for both spontaneous (Figure 4.15A) and oxytocin-induced (Figure 4.15B) experiments. At very small concentrations (10 pM and above), it was observed that the contractions were abolished (Figure 4.15A-B). Therefore, single concentrations (10pM and 30pM) were used to determine the effect on contractions (Figure 4.15C-D). It was observed that contractions were slowly decreased and if left for a long time, completely abolished.

As depicted in Figure 4.15, nifedipine was shown to be time-dependent on the strips and once completely abolished; its effect was not reversible even after one hour of washout.

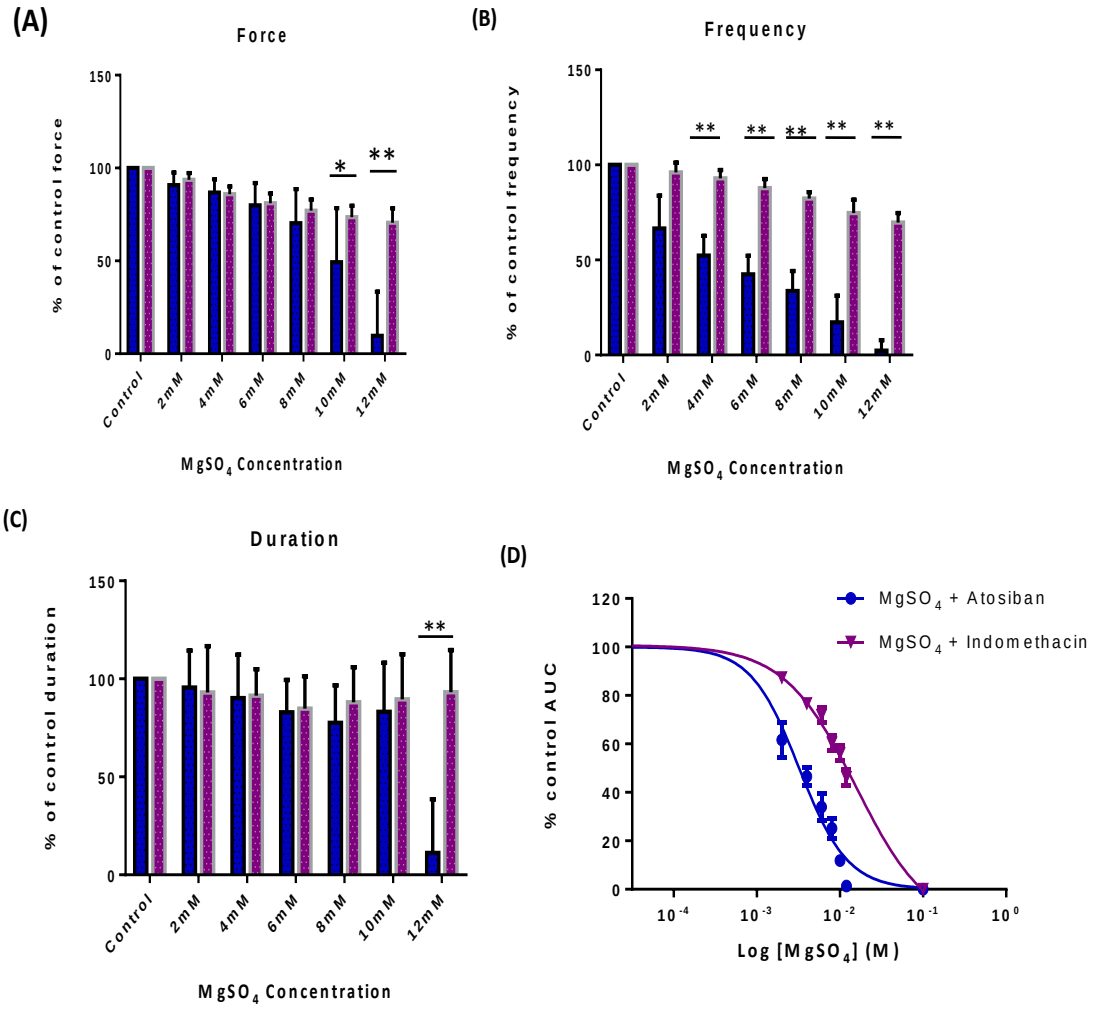


Figure 4. 15: A comparison of $MgSO_4$ plus Atosiban and $MgSO_4$ plus Indomethacin on oxytocin-induced contractions

Mean data ± SEM showing the concentration-dependent effect of $MgSO_4$+ Atosiban (blue) and $MgSO_4$ + Indomethacin (purple) in **(A)** force, **(B)** frequency, **(C)** duration, and **(D)** AUC of oxytocin-induced contracting strips. $MgSO_4$+ Atosiban significantly shifts the curve to the left ($P<0.001$) indicating less magnesium to inhibit contractions compared to $MgSO_4$ + Indomethacin. A significant difference in activity was found using ANOVA with Bonferroni post hoc. *denotes $p<0.05$, ** $p<0.0001$

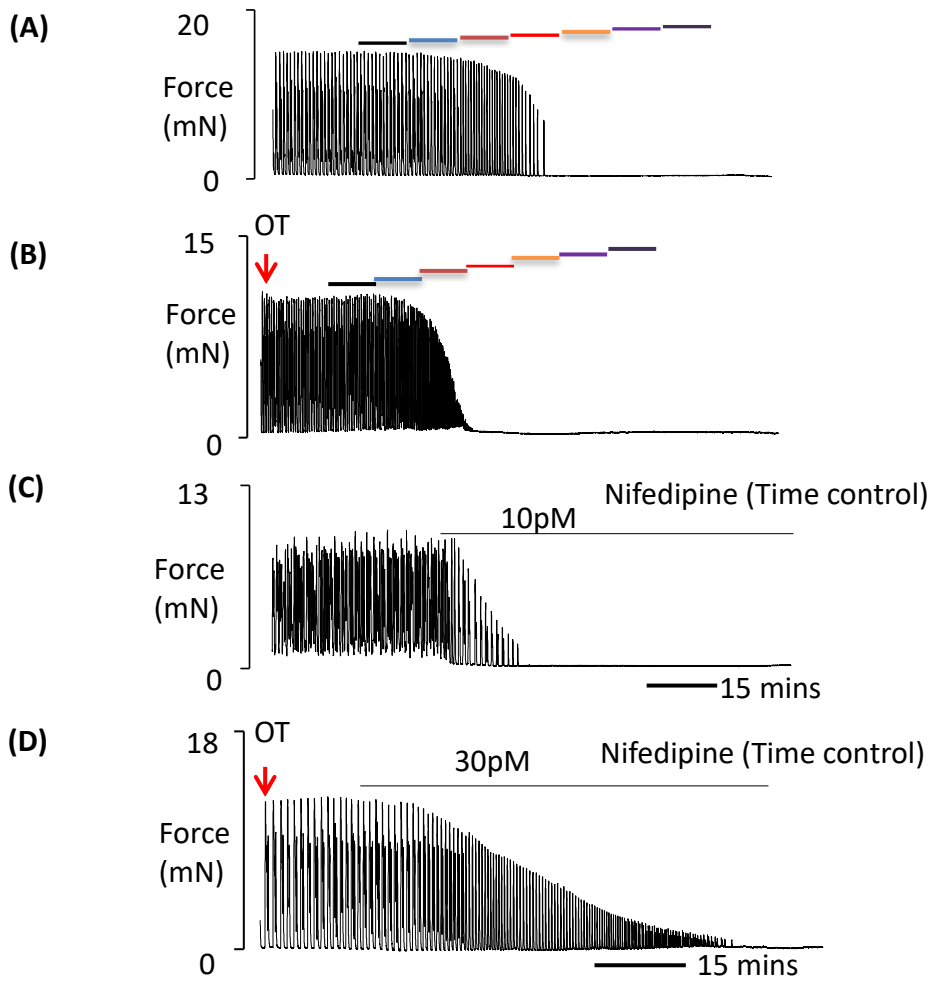


Figure 4. 16 The effect of nifedipine on uterine contractions of term pregnant mouse.

Representative isometric traces showing the effect of increasing concentration of nifedipine on (**A**) spontaneous contractions, and **(B)**, oxytocin-induced contractions). (**C** and **D**) showed the effect of a single concentration of nifedipine on contractions. With a single concentration, nifedipine reduced both spontaneous and oxytocin-induced contractions over time until contractions were completely abolished.

The short coloured bars indicate the 15 minute period where increasing concentrations of nifedipine were added- 3pM (black), 10pM (blue), 30pM (orange), 0.1nM (red), 0.3nM (green), 1nM (purple) and 3nM (brown).

For spontaneous contractions, the time-dependent effect of 10pM nifedipine is depicted in the bar charts (Figure 4.16A- D). Over a 1-hour period of nifedipine's application, there was a time-dependent reduction in force of contractions which reach significance at 40 minutes and higher. Although there was an observed reduction in frequency and duration of contractions, this didn't reach significance as the error bars were very high, resulting from a small n value (n=2), however, the AUC was significantly reduced at 30 minutes and higher.

Bar charts showing the time-dependent effect of 30pM nifedipine on oxytocin-induced experiments (n=4) are represented in Figure 4.17A-D. Nifedipine caused a significant reduction of contractions in force at 20 minutes and higher. The duration was also significantly reduced at 30 minutes and higher. The frequency of contractions was significantly increased at 50 minutes and higher. The overall activity (AUC) showed a significant decrease at 20 minutes and higher.

As a result of the time-dependency of higher nifedipine concentrations, I, therefore, chose a lower concentration of nifedipine (3pM) to be combined with the IC_{50} values of Mg for spontaneous (4mM) and oxytocin-induced contractions (10mM). At this concentration, contractions remained stable for a long period of time. Also, the concentration-dependent experiments were not carried out with nifedipine.

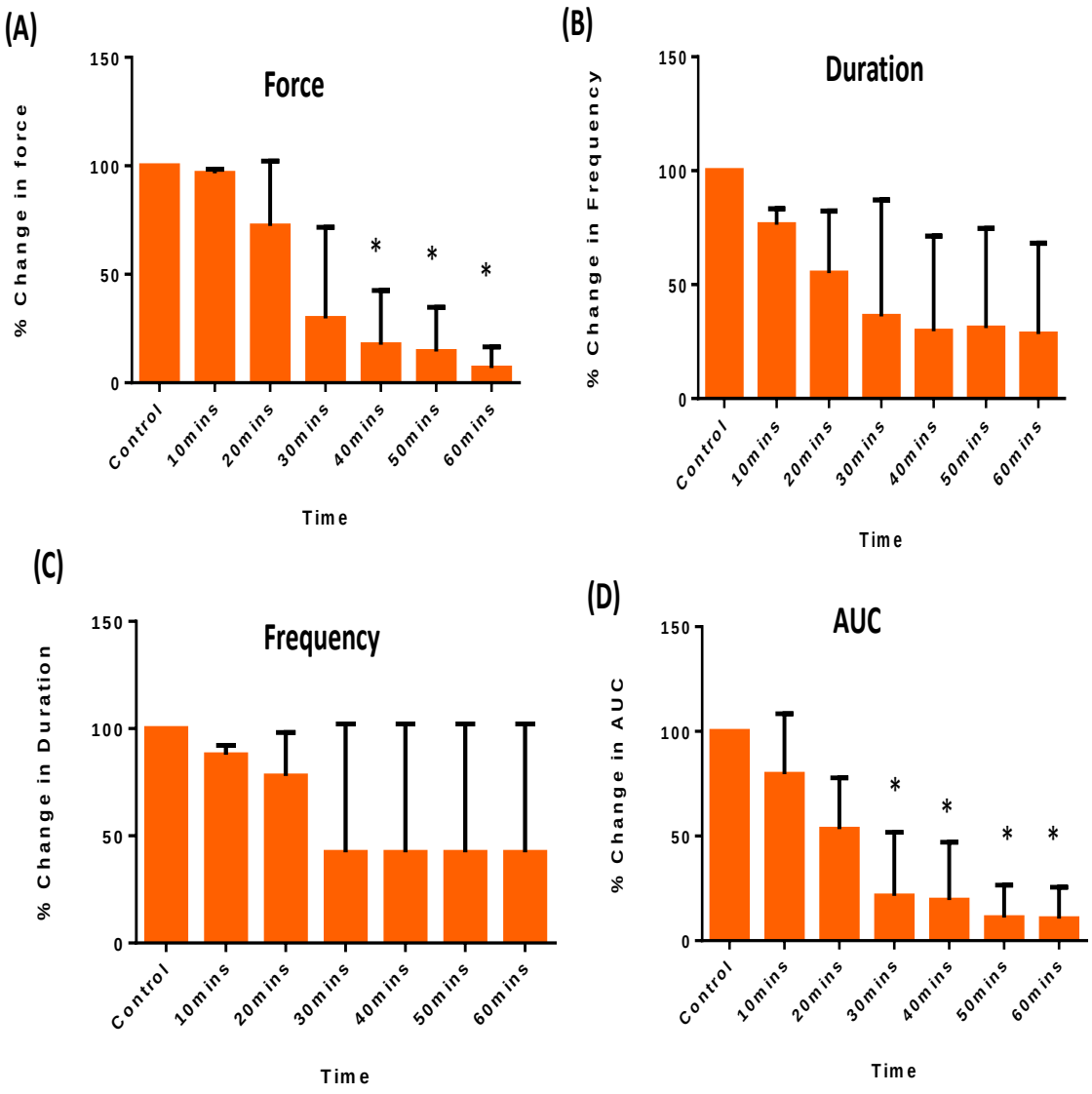


Figure 4. 17: Time-dependent effect of nifedipine on spontaneous contractions

Bar charts showing the time dependent effect of 10pM nifedipine on spontaneous contractions of term pregnant mouse during a 1-hr period of application. Nifedipine gradually decreased (A) force, (B) frequency, (C) duration and (D) AUC

Data represents mean values ± SEM (n=2). The asterisks on the vertical bars indicate a significant difference compared to control (ANOVA, $p < 0.05$). * denotes $p<0.05$.

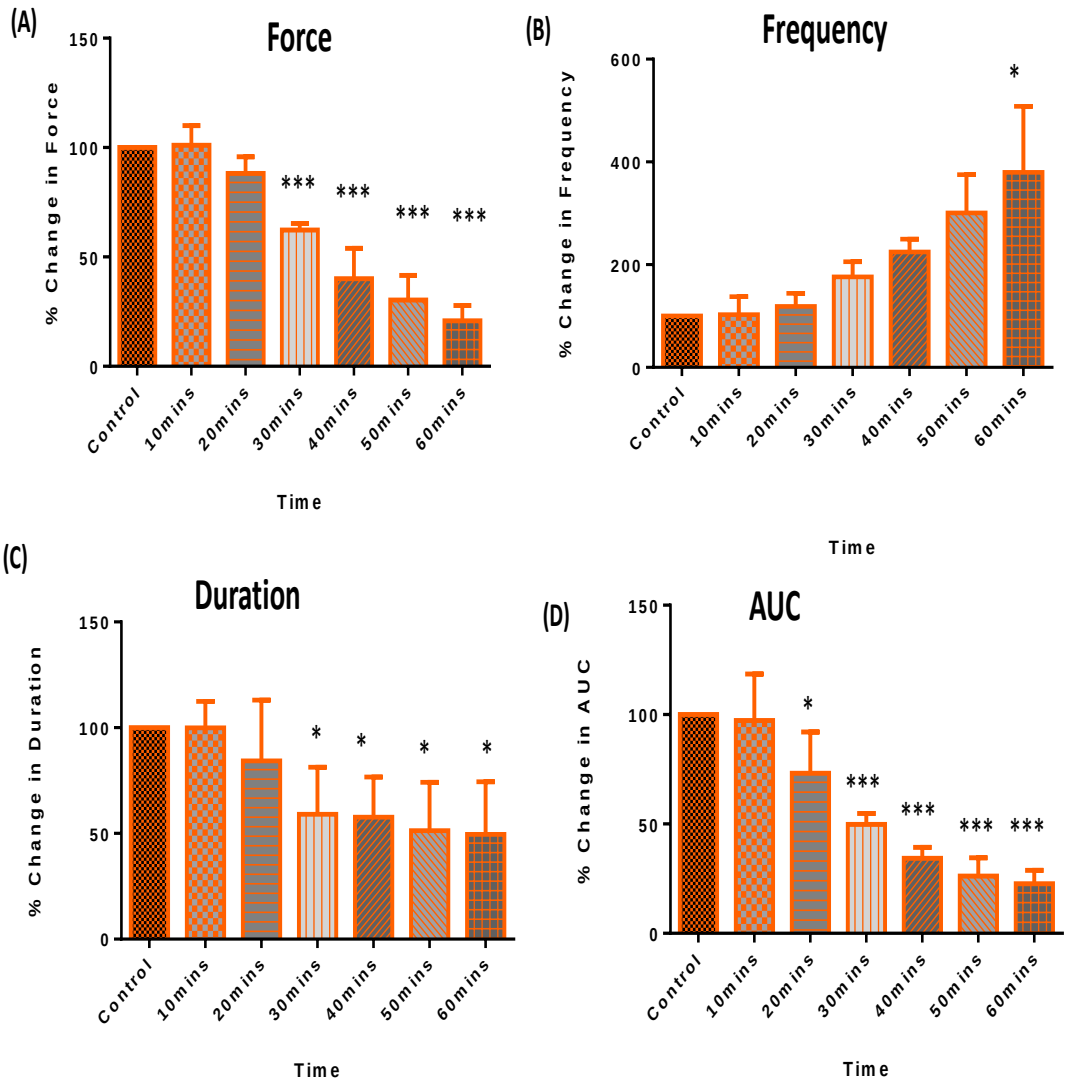


Figure 4. 18: Time-dependent effect of nifedipine on oxytocin-induced contractions

Bar charts showing the time dependent effect of 30pM nifedipine on oxytocin-induced contractions of term pregnant mouse during a 1-hr period of application. Nifedipine caused a change in (A) force, (B) frequency, (C) duration and (D) AUC.

Data represents average mean values ± SEM (n=4). The asterisks on the vertical bars indicate a significant difference compared to control (ANOVA, $p < 0.05$). * denotes $p<0.05$, *** $p<0.0001$.

4.4.7 The Effect of Mg plus nifedipine on uterine contractions of term pregnant mouse.

Having established a robust protocol (4.4.6), I proceeded to examine the effect of combining Mg + nifedipine in both spontaneous and oxytocin-induced contractions (Figure 4.18). Unlike in atosiban and indomethacin experiments, increasing concentrations of Mg have not used in Mg + Nifedipine experiments for reasons explained in section 4.4.6.

In spontaneous contractions (n=7), 4mM Mg + 3pM nifedipine reduced the force of contractions to 62.1% ± 14.0 of control, however, this reduction did not reach statistical significance. When this effect was compared to that seen with 4mM Mg alone (86.5% ± 6.5), there was again no significant difference (P=0.06). The frequency of contractions, however, was affected by Mg + nifedipine causing a significant reduction (56.2% ± 4.5, P<0.01) compared to control (100%). There was, however, no significant difference when the effect of the combination was compared to Mg alone (P=0.07). The duration of contractions was not significantly affected (91.3% ± 26.2, P =0.76). The AUC of contractions was significantly reduced to 46.4% ± 6.7 of control (p<0.001) while Mg alone caused the AUC to be significantly reduced to 64.6% ± 5.6 of control (p<0.001). Bar charts to represent the mean percentage compared to control ± SEM for force, frequency, duration and AUC are shown in Figure 4.19 A-D.

To determine the tocolytic efficacy of the combination of Mg + nifedipine under the hormonal influence of oxytocin, the effect of Mg (10mM) + nifedipine (3pM) was also examined in oxytocin-induced contractions (n =6). In the presence of oxytocin, Mg + nifedipine still produced a decrease in myometrial contractions: The force of contractions was significantly reduced to 61.5% ± 6.3 of control (P<0.01) while Mg alone reduced the force to 81.1% ± 5.1 (P<0.05) of control. The frequency of contractions was significantly reduced b to 50.4% ± 10.1 (P<0.01). The duration of contraction was not significantly reduced with either Mg alone (P=0.69) or in combination with nifedipine (P= 0.72). The AUC was significantly reduced by Mg + nifedipine to 34.6% ± 6.8 compared to control (P<0.001). When the effect of Mg in

the presence of nifedipine was compared to that of Mg alone (51.0% ± 7.7, $P<0.01$), there was a significant difference ($P<0.05$). Figure 4.20 (A-D) shows bar charts to represent the percentage change in force, frequency, duration and AUC of contractions.

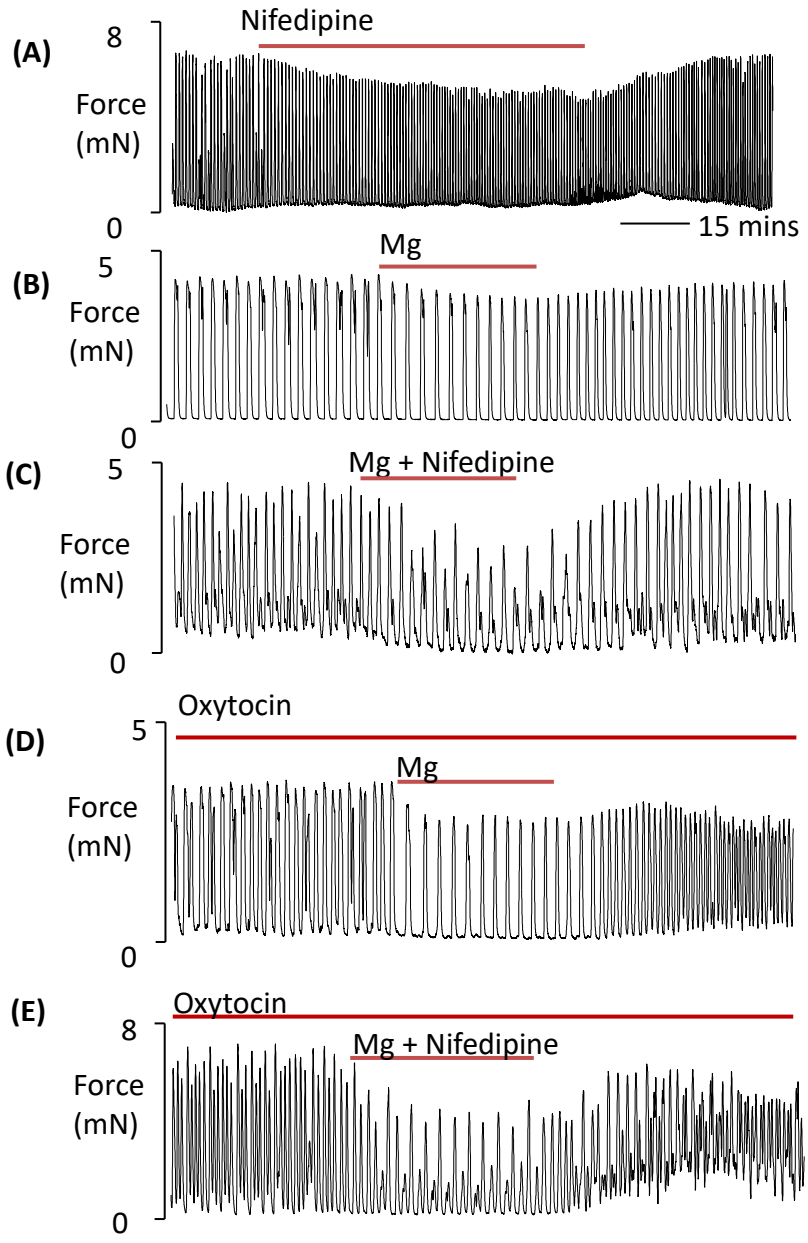


Figure 4. 19: The effect of $MgSO_4$ plus Nifedipine on spontaneous and oxytocin-induced contractions

Isometric traces showing **(A)**, the effect of 3pM nifedipine on contractions; (B), the effect of 4mM Mg alone on spontaneous contractions; (C) the effect of 4mM Mg and 3pM nifedipine on spontaneous contractions; (D), the effect of 10mM Mg on oxytocin-induced contractions and (E) the effect of10mM Mg and 3pM nifedipine on oxytocin-induced contractions.

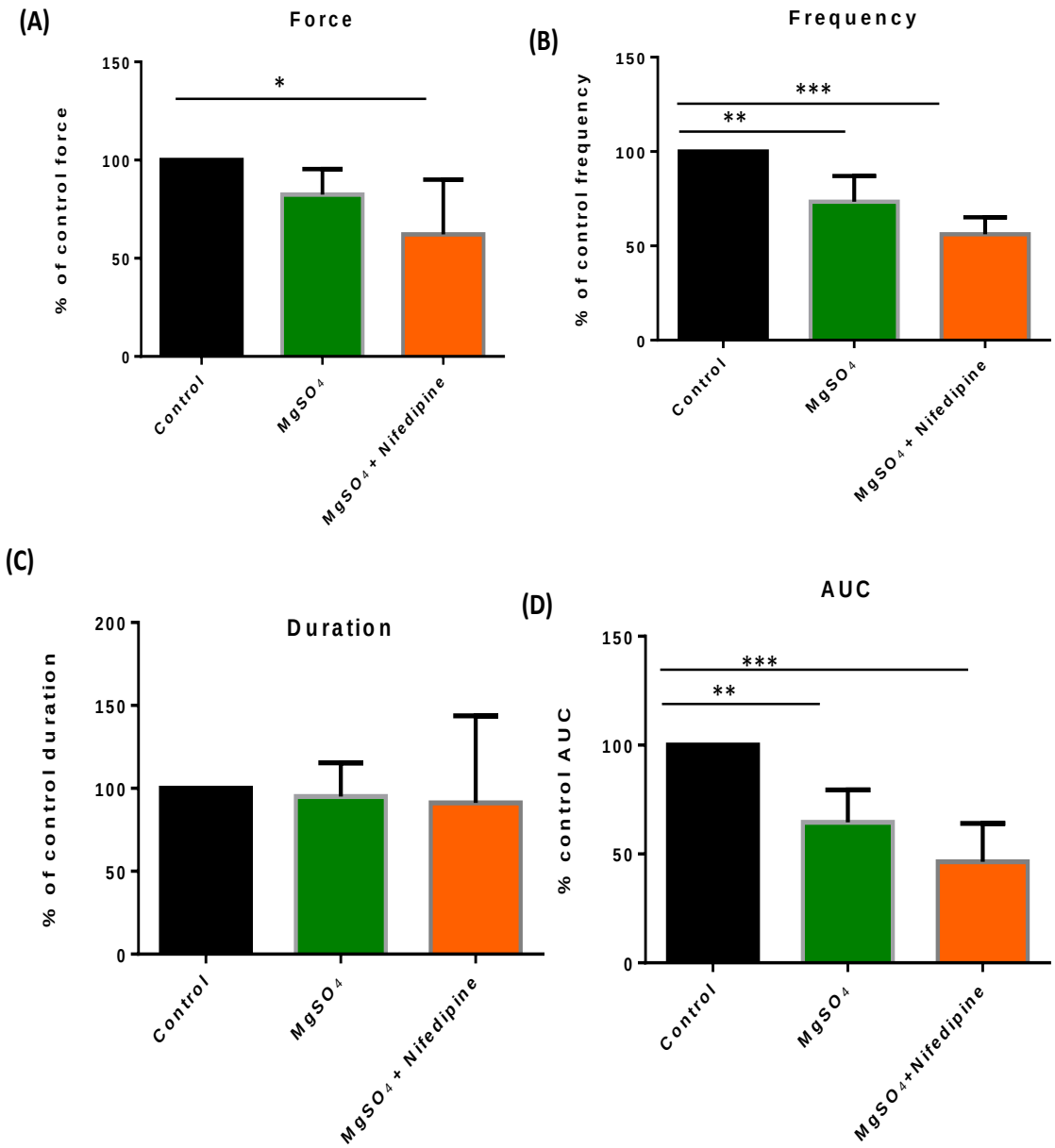


Figure 4. 20: The effect of $MgSO_4$ and $MgSO_4$ plus Nifedipine on spontaneous contractions.

Bar charts showing the effect of $MgSO_4$ alone (green) and in combination with nifedipine (orange) on (A), force (B) frequency, (C) duration and (D) AUC of spontaneous contractions. The difference in activity was found by ANOVA using Bonferroni post hoc test. *denotes $p<0.05$, ** $p<0.01$ ***$p<0.001$.

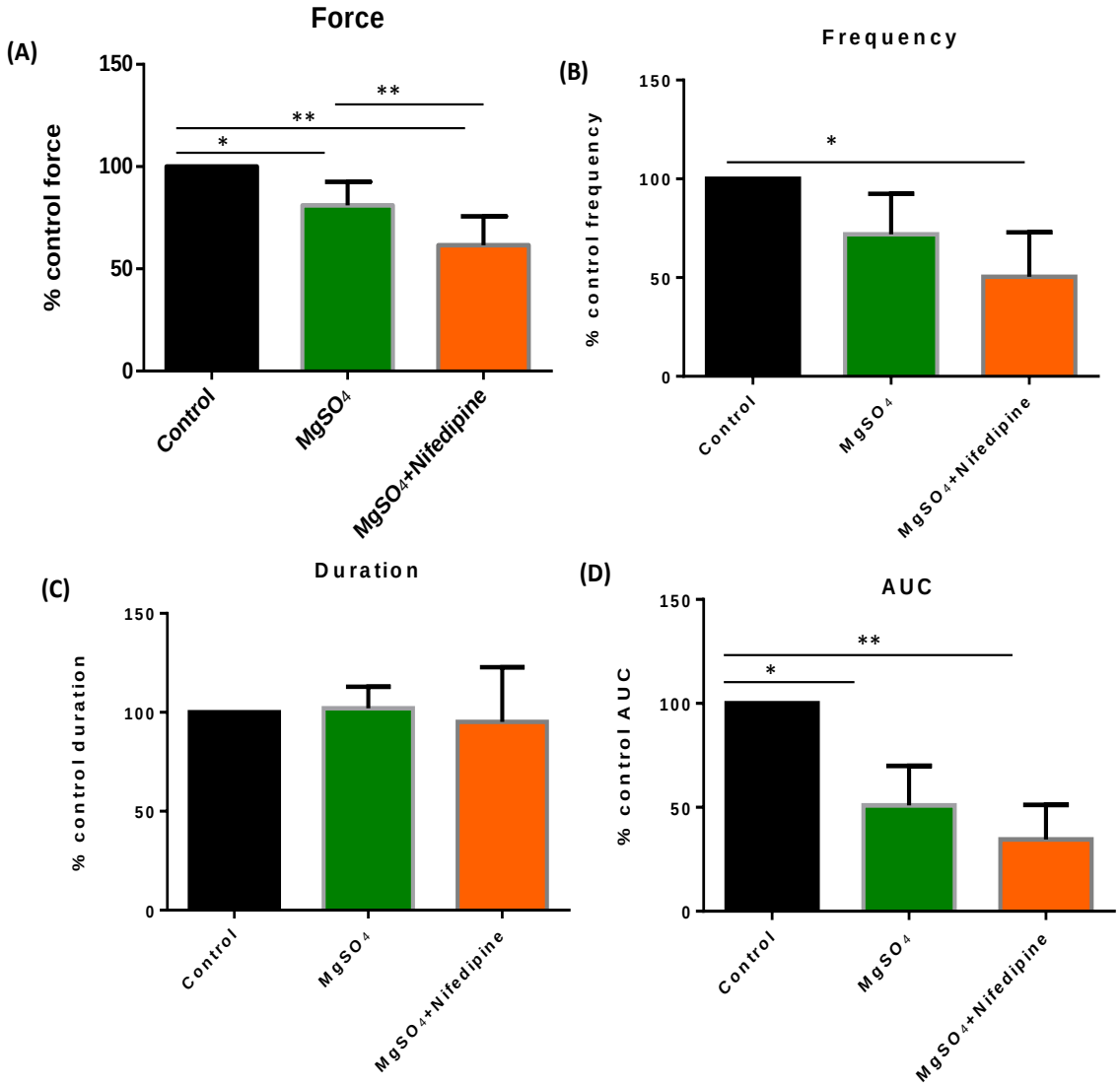


Figure 4. 21: The effect of $MgSO_4$ and $MgSO_4$ plus Nifedipine on oxytocin-induced contractions.

Bar charts showing the effect of $MgSO_4$ alone (green) and in combination with nifedipine (orange) on (**A**), force **(B)** frequency, **(C)** duration and **(D)** AUC of oxytocin-induced contractions. The difference in activity was found by ANOVA using Bonferroni post hoc test. *denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.4.5 Comparison between combined tocolytics

To determine which tocolytic in combination with Mg is most efficacious, I, therefore, chose one concentration of Mg (10mM) which was used in all procedures and in the presence of oxytocin which could, therefore, be used to compare responses across the different tocolytic combinations with Mg. (Figure 4.21).

Mean data for Mg and the combinations on all contractile parameters of contractility were plotted together and statistical analysis between groups performed. In the force of contractions (Figure 4.21A), there was a significant difference between all groups ($p<0.05$). There was also a significant difference between all groups in frequency (Figure 4.21B) and AUC (Figure 4.21D) of contractions ($p<0.0001$). The duration of contractions (Figure 4.21C) between groups did not reach statistical significance ($p=0.57$). The mean values ± SEM for all parameters are represented in Table 4.5. My data suggests the order of combined tocolytic potency to be: Mg + atosiban > Mg + nifedipine > Mg + indomethacin.

4.4.6 The Effect of Acidification on the efficacy of combined tocolysis.

Since my investigations revealed that the most effective combination of tocolytic with magnesium is Atosiban, I wanted to investigate how acidification could affect the efficacy of this combination. To determine the effect of extracellular acidification (pH_o), the pH of the Mg + atosiban solution (pH 7.4) was changed to an acidic solution (pH 6.9). This was applied for 15 minutes and the change in contractile properties compared to pH 7.4 was measured. A typical trace is shown in Figure 4.22A. All contractions were induced with 0.5nM oxytocin while 4mM Mg plus 500nM atosiban were used as inhibitors.

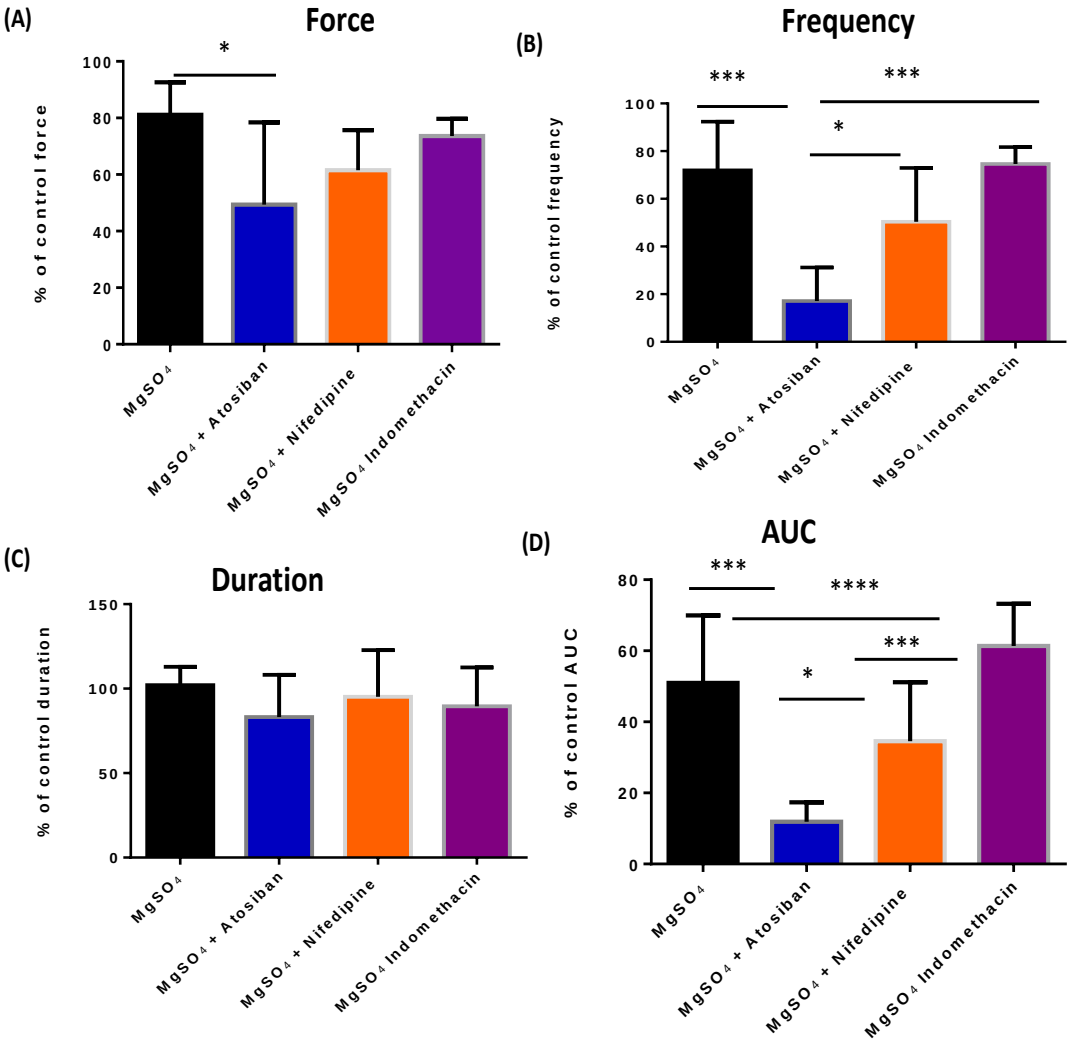


Figure 4. 22: The effect of $MgSO_4$ alone and in combination with Atosiban, Indomethacin and Nifedipine on oxytocin-induced mouse myometrium.

Bar charts showing the effect of $MgSO_4$ alone (black) and in combination with atosiban (blue), nifedipine (orange) and indomethacin (purple) on (A), force (B) frequency, (C) duration and (D) AUC of oxytocin-induced contractions. The difference in activity was found by ANOVA using Bonferroni post hoc test. *denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$.

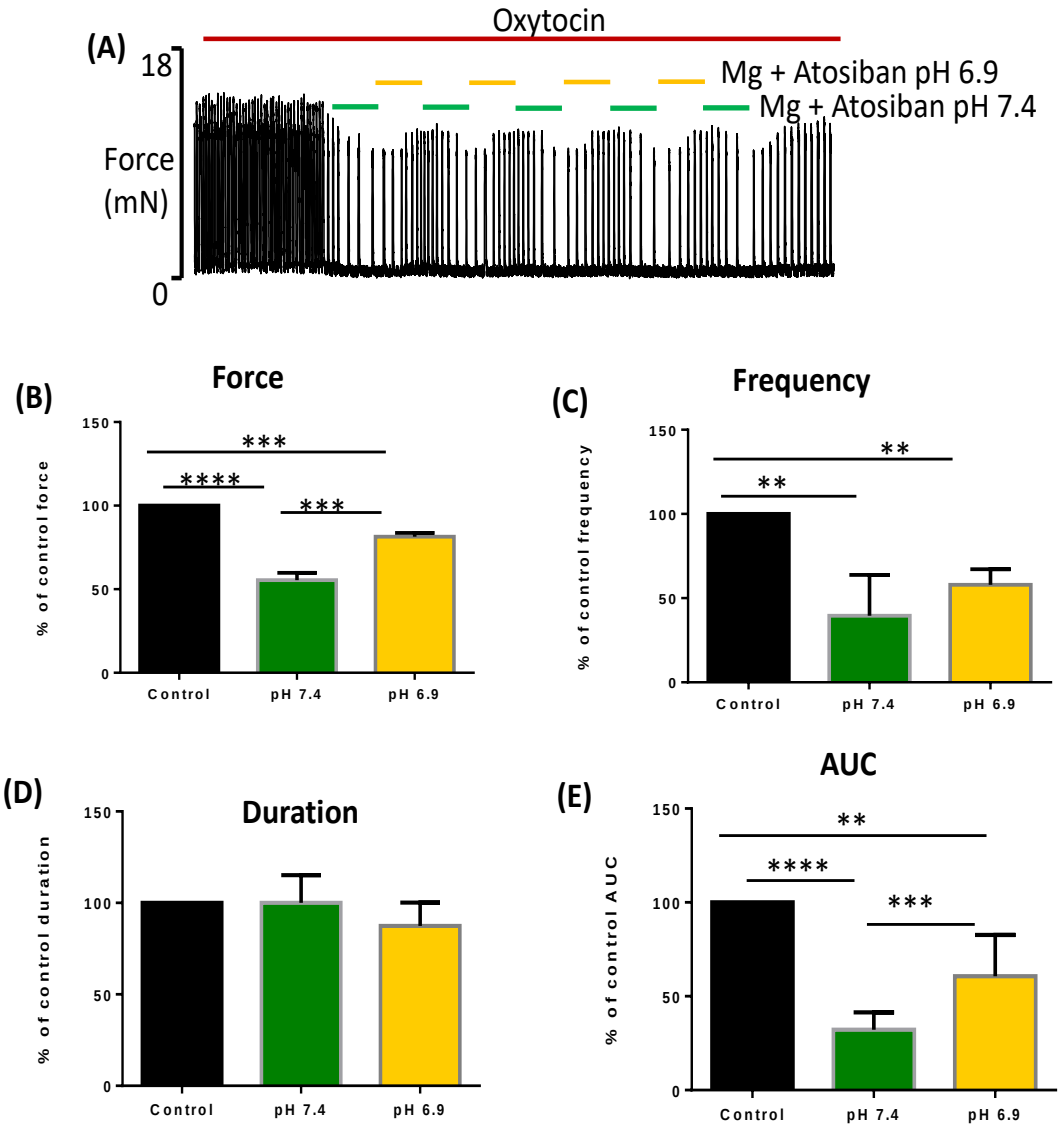


Figure 4. 23: The effect of extracellular acidification on $MgSO_4$ plus atosiban

(**A**) Representative isometric recording showing the effect of $MgSO_4$ + atosiban on oxytocin-induced contracting strips at pH 7.9 and 6.9 (n=8). Bar charts show the effect of extracellular acidification on $MgSO_4$ + atosiban. Mean data ± SEM (denoted by error bars) showing decrease in **(B)** amplitude **(C)** duration **(D)** frequency **(E)** AUC in response to pH 7.4 (green) and pH 6.9 (yellow) when compared to the control (black). Significant difference was calculated using ANOVA. ** denotes p<0.01, *** P<0.001, ****P<0.0001.

It was observed that acidification caused an increase in force, frequency and AUC of contractions resulting in reduced efficacy of the combination. Analysis of AUC values showed significant differences between Mg + atosiban (pH 7.4) compared to control (32.2% ± 3.5, n=7; $p<0.0001$) and Mg + atosiban (pH 6.9) compared to control (60.7% ± 8.3, n=7; $p<0.01$). Comparison between the effects of the two groups (pH 7.4 and pH 6.9), showed the reduction in activity to be significantly greater under pH 7.4, P=0.005. Bar charts comparing the effect before and after acidification in comparison to control are shown in Figure 4.22 B-E.

4.5 Discussion

Tocolytics have been shown to relax myometrial contractions and they act via different mechanisms. Many studies including a Cochrane review (Vogel et al., 2014a) conclude that the combination of tocolytics, especially those affecting different pathways, could have a greater effect compared to single agents and could also reduce dosage used, thereby reducing side effects.

To the best of my knowledge, this chapter is the only study that has extensively investigated the effect of Mg in combination with the most commonly used tocolytics (indomethacin, atosiban and nifedipine) in pregnant mouse uterus. This study was conducted to investigate the inhibitory effect of individual tocolytics, investigate the effect of combining Mg with these tocolytics and to examine how extracellular acidification and hormonal influences such as oxytocin could affect its efficacy. My data showed that all drugs studied (Mg, atosiban, indomethacin and nifedipine) individually had a tocolytic effect *in vitro* on pregnant mouse uterus.

The effect of indomethacin on uterine contractility

This experiment showed that indomethacin has a concentration-dependent inhibitory effect on mouse uterine contractions which confirms work previously described in human myometrium (Baumbach et al., 2012). Indomethacin arrests and completely inhibits spontaneous contractions, with complete abolition of contractions at 100 μM.

To mimic the hormonal milieu in labour, the effect of indomethacin on oxytocin-induced contractions was also investigated. As shown in Table 4. 2, the effects of indomethacin were less in the presence of oxytocin compared to spontaneous contractions, which may be due to the increased contractile activity with oxytocin stimulation. These data also revealed that the response to indomethacin under oxytocin is shifted to the right, indicating a greater concentration needed to suppress oxytocin's effect on contractions. At higher concentrations, indomethacin drastically abolished contractions, suggesting that indomethacin may be acting via another mechanism other than inhibiting COX and prostaglandin production. Similar rapid inhibition was found in human myometrium by our group (Arrowsmith et al.,

2016) and others (Sawdy et al., 1998). They suggested that indomethacin may partly act via calcium channel blockade, thereby reducing calcium entry (Sawdy et al., 1998). They and others (Vane and Williams, 1973, Riendeau et al., 1997) also showed that indomethacin had more of an inhibitory effect on contractility than on calcium currents, suggesting that calcium blockage may be one of several mechanisms by which indomethacin acts. In this study, indomethacin only arrested contractions at >30 µM, which is higher than the therapeutic plasma concentrations (1-2 µM). It is however uncertain if this effect of indomethacin is of any relevance *in vivo*.

The effect of atosiban on uterine contractility

Under oxytocin stimulation, atosiban caused a concentration-dependent reduction in contractions. The force and frequency of contractions were affected, including the force integral which takes into account the whole contractile activity. At the highest concentration used (3µM), atosiban inhibited contractions to less than spontaneous activity. This may be as a result of atosiban's agonistic action on oxytocin and vasopressin receptors or perhaps by an unknown mechanism. Similar response to atosiban has been shown in term pregnant women (Büscher et al., 2001).

Atosibans's effect seemed more potent in the frequency than force of contractions.

The effect of nifedipine on uterine contractility

This study has shown that nifedipine significantly inhibits spontaneous and oxytocin-induced contractions even at very low concentration (10pM). This finding supports other published work confirming nifedipine as a potent tocolytic (Moynihan et al., 2008b, Forman et al., 1982). This study is the first to show a time-dependent inhibitory effect of nifedipine on mouse uterine contractility. Due to the inhibitory effect observed at very low concentrations, it is clear that nifedipine may be a potent tocolytic.

My data clearly reveals that nifedipine was able to inhibit contractions even when induced by oxytocin. This may partly be as a result of one of oxytocin's mechanisms

which involve depolarizing and increasing calcium entry via the L-type calcium channel (Wray, 2007). There was greater tocolytic effect of nifedipine in spontaneous contractions than in oxytocin-induced, which may be due to the increased uterine activity with the stimulant.

Spontaneous uterine contractions are produced by changes in membrane potentials resulting in the burst of action potentials (Kawarabayashi et al., 1989), and L-type calcium channel opening (Wray et al., 2003, Shmigol et al., 1998). My data shows reduced force and duration of contractions, which indicates a decrease in intracellular calcium caused by inhibition of L-type calcium channels. In spontaneous contractions, a decrease in frequency was also noted suggesting that the pacemaker activity is affected. When stimulated with oxytocin however, an increase in frequency was observed which may suggest that there is a quick recovery of the membrane to resting potential in the presence of oxytocin. Regardless of the increase in force and decreased force and duration of oxytocin-induced contractions, the overall contractile activity (AUC) was still reduced.

The effect of combined tocolytics

According to the NICE guidelines 2011, women who are at risk of preterm birth before 30 weeks of pregnancy are to be administered $MgSO_4$ to help reduce the risk of cerebral palsy. So, although $MgSO_4$ is not currently used for the treatment of preterm labour, it is being administered clinically in combination with a tocolytic which in the UK could either be indomethacin, nifedipine, or atosiban. This means that in certain preterm cases (<30 weeks), a dual tocolytic therapy may be given.

The idea of combined tocolytics has been studied in animals and human. Nifedipine has been studied in combination with ritodrine (Carvajal et al., 2017), β-adrenergic agonists (Hajagos-Toth et al., 2010), sildenafil citrate (Chiossi et al., 2010); indomethacin has been studied in combination with nifedipine (Kashanian et al., 2019), and ritodrine (Katz et al., 1983); while atosiban has been studied in combination with nimesulide (Grigsby et al., 2000), and magnesium in human myometrium (Arrowsmith et al., 2016). Nonetheless, there are only few studies that have investigated the effect of dual tocolysis involving $MgSO_4$ in combination

with atosiban, indomethacin or nifedipine, and comparing the inhibitory effects of the combination. In fact, a recent Cochrane review pointed out the lack of combination studies which involved the widely used tocolytics (Vogel et al., 2014a). Therefore, finding the best combination could help halt the increasing rate of preterm birth. However, owing to the time-dependent effect of nifedipine on myometrial contractions and atosiban having no effect on spontaneous contractions, different experimental procedures were used in the three combinations examined; Mg + atosiban, Mg + Indomethacin, and Mg + nifedipine.

This study shows the effect of each tocolytic alone and in combination with Mg. The combination of Mg with indomethacin caused a concentration-dependent decrease in mouse myometrial contractility in both spontaneous and oxytocin-induced contracting tissues, but less reduction was seen in the presence of oxytocin. The tocolytic effect on the spontaneously contracting tissue was additive, however, in oxytocin induced contractions; the effect on contractions was antagonistic as the combination reduced the tocolytic effect of both drugs individually. Indomethacin, which is a non-selective COX inhibitor, acts by blocking prostaglandin production and hence reduces uterine stimulation, while $MgSO_4$ acts by hyperpolarising the plasma membrane and competing with extracellular calcium or entry leading to a reduction in the availability of calcium required for myosin light chain kinase activation, thereby reducing myometrial contractility (Simhan and Caritis, 2007). This finding supports the finding by (Vane and Williams, 1973) who investigated the effect of indomethacin on both non-pregnant and pregnant rat uteri and reported that in pregnant myometrium, oxytocin-induced contractions were not antagonized by indomethacin but caused a significant reduction in the spontaneous contractions.

With Mg + atosiban combination, a concentration 3 times higher than Mg alone was needed to obtain the same effect as seen with the combination. This indicates that the effect of $MgSO_4$ + Atosiban on myometrial contractions was synergistic. This result is similar to previous findings from our lab which investigated the effect of $MgSO_4$ and atosiban on strips obtained from term pregnant women undergoing caesarean section (Arrowsmith et al., 2016). This combination

potentiates the inhibitory effect of each drug on its own. This may be as a result of the two drugs acting via different intracellular pathways which enhance the inhibitory effects of each other. Magnesium interferes with the Ca^{2+} entry into the cell while atosiban plays a significant role as an antagonist at the site of the oxytocin receptors. However, it should be noted that atosiban had little if any effect on spontaneous contractions in humans (Arrowsmith et al., 2016) and hence the effect of atosiban on spontaneous contractions in mouse was not tested.

The combination of Mg and nifedipine has been reported to cause high side effects, owing to the potential interaction leading to hypotension and neuromuscular blockade (Magee et al., 2011). This combination has however been investigated in this chapter. Despite the maternal and neonatal side effects that result from dual tocolytic therapy, the NICE guidelines on preterm labour and birth (November 2015) provided no information on the safety of using $MgSO_4$ in combination with a tocolytic especially nifedipine which acts via a similar mechanism (RCOG, 2016), hence the need for this study.

In both spontaneous and oxytocin-induced contractions $MgSO_4$ + Nifedipine significantly inhibited contractions. When the combination was compared to magnesium alone, the difference did not reach statistical significance. This was true for both spontaneous and oxytocin-induced contractions.

The effect of Mg plus Atosiban and Mg plus Indomethacin on oxytocin-induced contractions was compared and there was a significant difference between them. Although the Mg plus Indomethacin combination caused a concentration-dependent decrease in contractile parameters, its effect was significantly lower than Mg + Atosiban. The concentration response curve for Mg + Indomethacin was shifted to the right as indicated by the IC_{50} values. When the effect of all three combinations were compared at a given Mg concentration, the greatest tocolytic effect was Mg plus Atosiban followed by Mg plus Nifedipine with Mg plus Indomethacin showing the least tocolytic effect.

As seen in Figure 4.1, there are 2 major pathways to contractility and the different tocolytics act on them to facilitate relaxation. Tocolytics may either stimulate

relaxatory pathways e.g. those involving cAMP signaling or inhibit stimulatory pathways, typically those involving IP3 formation or calcium (Kuć et al., 2011). IP_3 stimulate the release of calcium ions from sarcoplasmic reticulum and increases the intracellular calcium concentration. Most tocolytics (e.g. magnesium, atosiban, nifedipine and indomethacin) would normally have an effect on the second pathway. Magnesium which is an L-type calcium channel blocker acts by inhibiting calcium entry, intracellular calcium release and cytosolic calcium oscillations (Phillippe, 1998). Nifedipine, an L-type calcium channel inhibitor acts also by blocking of the inflow of calcium ions from extracellular stores, which is independent of IP3 pathway. Atosiban, an oxytocin receptor antagonist, prevents IP3 pathway stimulation by competitively inhibiting oxytocin binding to myometrial cell receptors. Atosiban binds oxytocin membrane receptor and directly blocks G-protein signalling. Through this mechanism, it inhibits inositol triphosphate synthesis. Indomethacin, a prostaglandin synthesis inhibitor blocks cyclooxygenase (COX) pathway. Prostaglandins are other stimulants which play a major role in maintenance of pregnancy and the beginning of labour. They also modulate myometrial contractions during labour as well as during reproduction (Hertelendy and Zakar, 2004)

The greatest tocolytic effect seen with Mg plus Atosiban may be as a result of the blockage of two different pathways which lead to increased intracellular calcium concentration. This combination results in inhibition of extracellular Ca^{2+} inflow via Mg and reduced calcium release from the SR. In addition, atosiban blocks IP3 synthesis and it blocks the cooperation with diacylglycerole in prostaglandin synthesis. Oxytocin and prostaglandin play major roles in influencing induction and sustenance of myometrial contractions. In these experiments, oxytocin was used to stimulate the contraction, hence the greatest inhibition was seen when Mg was combined with Atosiban. The least tocolytic effect seen with Mg plus Indomethacin may be because of oxytocin's effect, as the combination caused great inhibition in spontaneous contractions. A study which investigated the effect of Indomethacin in pregnant rats showed that Indomethacin did not suppress myometrial contractions in the presence of oxytocin (Fuchs et al., 1976). The inhibitory effect seen with Mg

plus Nifedipine was additive and this may be as a result of the blockage of the influx of extracellular calcium. Nifedipine and Mg act via similar mechanism and in addition Mg increases cAMP level which reduces intracellular calcium (Ben-Ami et al., 1994). Intracellular magnesium reduces calcium available for contraction by stimulating calcium dependent ATPase. Because of the similarity in mechanism of action between Mg and nifedipine, there have been questions regarding the safety of using both drugs in combination. Thorp et al demonstrated in isolated rat heart model that nifedipine caused a concentration dependent myocardial depression which increased the effect of magnesium (Thorp et al., 1990). Other studies (Snyder and Cardwell, 1989) have shown similar findings pointing to increased incidence of magnesium-related neuromuscular blockade and other maternal side effects. Other studies however have also demonstrated that the drug combination does not increase the risk of neuromuscular weakness and blockade (Magee et al., 2005).

Effect of pH change on tocolytic efficacy

Changes in extracellular and intracellular pH alter uterine contractions in guinea pigs, rats and women (Parratt et al., 1994, Taggart and Wray, 1993, Naderali et al., 1997). Despite the fact that pH changes have been widely studied in the uterus, the regulatory mechanism at the level of ion channels is poorly understood. This section of the study was designed to establish the effect of extracellular acidification and its influence on tocolytic efficacy. Previous reports in rats and human have shown acidification to decrease contractions while alkalinisation increased contractions (Parratt et al., 1994, Taggart and Wray, 1993). My data however, indicates that extracellular acidification produces a marked increase in the force and frequency of myometrial contractions, hence reducing the tocolytic efficacy of Mg plus Atosiban. This result is consistent with an increase in contractile force observed in non-pregnant guinea pig (Naderali and Wray, 1999b) and an increase in contractions of uterine circular muscle of pregnant mice (Hong et al., 2016). Previous study from our lab (Almohanna et al., 2016) that investigated the effect of pH_o on mouse myometrium also showed that reduced pH_o caused modulation of force and frequency of spontaneous contractions. This increase was seen in non-pregnant, mid-term (day 14) and term-pregnant (day 19) mice myometrium.

Although pH is thought to act via different mechanisms, one possible mechanism by which extracellular acidification stimulates force thereby reducing tocolytic efficacy may be via the displacement of Ca^{2+} from intracellular binding sites (Parratt et al., 1995). It is known that protons and Ca^{2+} compete for intracellular binding sites (Wray, 1988), hence a rise in H^{+} may lead to an increase in free $Ca^{2+.}$ This mechanism may partly explain the reduced efficacy of tocolytics used in clinics. It has been shown that changes in pHo can slowly cause changes in pHi and affect cellular function (Austin and Wray, 1995, Wray, 1993, Fry et al., 1994); however, this interaction with each other makes it difficult to distinguish their effects (Naderali and Wray, 1999a).

In summary, the data in this chapter shows the effect of magnesium sulphate in combination with atosiban, indomethacin or nifedipine on mouse myometrium and its effect on mouse myometrium. The data shows that magnesium plus atosiban was more potent in reducing myometrial contractions compared to the other combinations, possibly due to the combination acting via different contractile pathways. The data also shows that extracellular acidification caused an increase in force and frequency of contractions in the presence of the combination (Mg plus atosiban) thereby reducing the efficacy of the combination. Further investigation into the mechanism involved in pH and tocolytics may shed further insights into these findings.

4.5.1 Limitations of this study

- The strips used in this study were obtained from non-labouring mice and since tocolytics are normally used in treatment of preterm labour, this data may not translate into clinical scenarios. Studies testing the drugs on preterm labouring mice would be helpful.
- There were no mechanistic studies investigating the effect of intracellular pH or calcium signaling in this study. These experiments would need to be done to fully understand the effects seen in my samples.

- In this study, experiments were not performed in human myometrium. It would be of great benefit is this work is carried out on human models and ultimately conducts pilot studies in women.
- There were no experiments carried out to determine the safety of the combinations. This would need to be done as there may be interactions between the drugs which could result in serious maternal and fetal side effects.

4.6 Conclusion

This study is the first to compare the tocolytic effect of Magnesium plus atosiban, magnesium plus indomethacin and magnesium plus nifedipine on uterine contractility of mouse.

In this chapter, I have shown that Magnesium plus indomethacin concentration dependently decreased spontaneous uterine contractions and this effect was overcome by the application of oxytocin. This suggests that indomethacin may not be the best tocolytic in cases where magnesium is administered.

I also investigated the effect of magnesium plus atosiban on oxytocin-induced contractions. The results showed that the combination caused a significant concentration-dependent decrease on oxytocin-induced uterine contractions compared to atosiban or magnesium alone. Using the IC_{50} values, the effect seen almost mirrored the effect of magnesium on spontaneous tissues (chapter 3), indicating that this combination could reverse the effect of oxytocin.

The effect of Mg plus nifedipine was also investigated in spontaneous and oxytocin-induced contractions. My data showed that the combination decreased contractility compared to magnesium or nifedipine alone.

When comparing all three combinations, magnesium plus atosiban showed to be the best tocolytic combination in my tissues, hence the effect of extracellular pH was examined on this combination. My results showed that pHo caused an increase in contraction therefore reduced the tocolytic efficacy of the combination.

This work suggests a better tocolytic effect with magnesium plus atosiban, nevertheless, the effect of certain factors that affect uterine environment during labour e.g. lactate production, hypoxia and acidification needs to be explored.

Chapter 5

The Expression of Calcium Channels at Different Gestational States of Mouse Uterus

Chapter 5

The Expression of Calcium Channels at Different Gestational States of Mouse Uterus

5.1 Abstract

In pregnant uterus, calcium influx is essential for excitation-contraction coupling and the L-type calcium channels play an important role in calcium influx as well as pregnancy and labour onset. This thesis is focused on magnesium and its effect on uterine contractility. Since Mg's main mechanism of action is via antagonism of calcium entry at the calcium channels, it is important to investigate whether calcium channel expression also changes throughout gestation. The gestational regulation of calcium channel expression in the uterus is not yet completely understood, and no previous studies have established the expression of L-type calcium channel in mouse at different gestational stages. Therefore, the aim of this chapter was to determine the expression of L-type calcium channel (Cav 1.2) in mouse non-pregnant, pregnant and postpartum uterine tissues. This chapter also determined stable reference genes for studying gene expression across mouse gestations.

Uterus samples were collected from C57BL/6 mice at different gestational states (non-pregnant, days 14, 18 and 19 of pregnancy and postpartum). Using end point polymerase chain reaction (PCR) the presence of the L-type calcium subunits (Cav 1.1, Cav 1.2 and Cav 1.3) was examined. The mRNA expression levels of T-type calcium channel were detected by quantitative Real time PCR. Using geNorm analysis, the most stably expressed reference genes from a panel of 12 genes were analysed.

This chapter showed that the presence of Cav 1.2 in mouse myometrium. The mRNA expression of Cav 1.2 increased as pregnancy progressed but didn't reach non-pregnant levels and was further increased after labour (postpartum).

5.2 Introduction

During labour, the uterus undergoes certain changes which enable it to contract and expel the foetus. Preterm labour is suggested to result from early contractions of the uterus. It is well known that contractions of uterine smooth muscles are dependent on the rise in the concentration of intracellular calcium (Floyd and Wray, 2007, Shmygol and Wray, 2005). In the uterus, the voltage gated calcium channels (VGCC) form the main vehicle for calcium entry (Young et al., 1993) Voltage gated calcium channels are transmembrane proteins which are sensitive to changes in cell membrane potential and play a major role in transportation of calcium ions into cells. They are formed as complex of different subunits: α_1, $\alpha_2\delta$, β, and γ. However, it is the α1 subunit which forms the Ca^{2+}selective pore containing the voltage-sensitive apparatus. The other subunits are thought to have important roles in channel gating. The α2δ subunit is composed of an extracellular α2 segment linked to a trans-membrane δ segment, which anchors the α2 segment (Felix et al., 1997, Klugbauer et al., 2003). Overall, the binding of the α2δ subunit results in increased $[Ca^{2+}]i$ and so again will promote myometrial contractility. The β-subunit binds with high affinity to the linker loop between repeats I and II in the α1-subunit (Bichet et al., 2000, Dolphin, 2003). It has been shown that binding of the α2δ- and β-subunit the to the α1 subunit modifies voltage dependence of both activation and inactivation (Bichet et al., 2000) (Klugbauer et al., 2003), current amplitude (Gurnett et al., 1996, Felix et al., 1997), number of drug binding sites and number of affinity drug binding (Gurnett et al., 1996).

There are a number of different types of VGCC including; neural N-type, P/Q-type, L-type, R-type and T-type channels. In the uterus, the two main types which have been identified include the L-type also known as dihydropyridine channel (dihydropyridine is located on the α1 subunit and characterize the channel) and T-type channels (Kamkin et al., 2006). In the L-type calcium channel family, four α subunits have been classified namely: α1S, (Cav 1.1), α1C, (Cav 1.2), α1D, (Cav 1.3) and α1F, (Cav 1.4).These 4 α subunits are known for their expression in skeletal muscle, smooth muscle, ventricular myocytes and neuronal dendrites respectively.

The T-type VGCC family consist of 3 α subunits: α1G, (Cav 3.1) α1H, (Cav 3.2), and α1I (Cav 3.3). T-type channels are known for their expression in neurons and cells with pacemaker activity. Cav 3.1 and Cav 3.2 have been shown to be expressed in rat myometrium (Ohkubo et al., 2005). They investigated the mRNA expression of the subunits in longitudinal and circular muscle layers, showing variation in both muscle layers.

A few studies have shown that as well as expressing these channels, there are also changes in L- and T-type channel expression during gestation in different species (Ohkubo et al., 2005, Collins et al., 2000, Zhang et al., 2015). In mouse, Zhang et al (2015) investigated the mRNA and protein expression of T-type α1G (Cav 3.1) and α_1H (Cav 3.2) and showed that both subunits were expressed in the myometrium and that the expression levels increased towards term followed by an abrupt decrease in labour. Another study examined the expression of the L-type α1C subunit throughout gestation in rats (Mershon et al., 1994). They showed a significant increase of DHP binding sites at day 14 compared to non-pregnant controls which remained elevated through labour, then decreased postpartum. Tezuka et al also showed in rat myometrium, a gradual increase in α_1 and β subunits throughout gestation followed by a sudden decrease during labour for α1, but an abrupt increase in the β subunit (Tezuka et al., 1995). In guinea pigs, Collins et al investigated the changes in gestational expression of α and β subunits of the L-type calcium channel (Collins et al., 2000). Their results show expression of α subunits at day 34 (approximately day 10 gestation in mouse) which increased towards term while the β subunits were barely detectable at days 34 or 47 but increased at day 54 and declined at day 64. Interestingly, others have also shown that calcium current density during pregnancy remains unchanged (Inoue and Sperelakis, 1991). With all the studies on the expression of L- and T-type calcium channels, this thesis focuses mainly on the L-type calcium channels as they are known to play a more significant role in pregnancy.

The gestational regulation of calcium channel expression in the uterus is not yet completely understood. This study is focused on Magnesium and its effect on uterine contractility. I have already shown differences in the efficacy of Mg on

myometrium from different gestational states (Chapter 3). Hence, given Mg's main mechanism of action is via antagonism of calcium entry at the calcium channels, it is important to investigate whether calcium channel expression also changes throughout gestation. I anticipate that this data will aid our understanding of the differences in contractile activity observed with Mg as described in chapters 3 and 4.

The first aims of this chapter therefore were to:

viii. determine if L-type calcium subunits (Cav 1.1,1.2 and Cav 1.3) are expressed across different gestations of the mouse uterus; and if present,

ix. quantify the expression of L-type calcium α subunits at different gestational states using real time qPCR.

Real-time polymerase chain reaction (quantitative PCR, qPCR) is well established as one of the most sensitive and reproducible quantification methods for gene expression analysis (VanGuilder et al., 2008). Although qPCR is used to determine the fold change of the expression of genes between samples, the selection of an internal control or reference gene(s) (previously known as housekeeping genes) is a crucial step in the experimental design process. Normalising the expression level of target gene/gene of interest to this control is essential to improve expression data accuracy and reliability. For example, it enables differences in the efficiency of reverse transcription between samples as well as differences in sample concentration in the final PCR reaction such as potential pipetting errors to be controlled for (Udvardi et al., 2008, Gutierrez et al., 2008).

The stability of several genes e.g. ACTB, GAPDH, 18S and HPRT in different species and tissues has already been experimentally determined and they have been widely used as reference genes in studies of gene expression. However, it is important that the stability of these genes is maintained across all experimental conditions including throughout gestation (Thellin et al., 1999, Bustin, 2000). To ensure reliable and repeatable results in gene expression experiments, in February 2009, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published (Bustin et al., 2009). A major key to

improving the fidelity of qPCR results of gene expression is by selecting appropriate reference genes (Vandesompele et al., 2002, Guo et al., 2014). Therefore, the final aims of this chapter were to:

x. examine the most commonly used reference genes in mouse uterus (all gestational states) as published in the literature; and
xi. identify the most stable reference genes for qPCR across different gestational states in the mouse.

5.3 Materials and Methods

5.3.1 Animals and uterine tissues

Adult non-pregnant (NP), time-mated (d14, d18 and d19) and postpartum (PP) mice were used in this chapter. Mice were humanely killed by a rising concentration of CO_2 anaesthesia followed by cervical dislocation. The uterus was isolated, cleaned of placentas and connective tissues (where necessary) and placed immediately in HEPES buffered Krebs solution. Other tissue types including brain, kidney, skeletal muscle and heart were also collected which were used as positive controls in some experiments. Tissues were weighed and placed in RNA later solution for 24 hours at 4°C before being frozen and stored at -70^{o}C until use.

5.3.2 RNA extraction

Tissues for each group (NP, 14d, 18d, 19d and PP) were pooled from at least 3 mice. Sixty to one hundred milligrams of tissue was homogenized in TriZol Reagent (Invitrogen Life technologies) using an ultratraxx homogenizer. RNA was extracted using a Trizol plus RNA purification kit (life technologies) which uses a silica-cartridge (column-based) method to bind and purify the RNA, from which it can be further washed to remove contaminants before being eluted in RNase-free water. RNA concentration and purity was determined by measuring the 260/280 and 260/230 absorbance ratios using a NanoDrop^{TM} 1000 spectrophotometer. To ensure the viability of the samples and reduced contamination, all samples used ranged between 2.0 and 2.2 for the 260/230 ratios and 260/280 ratios. RNA was stored at -70^{o}C until used subsequently for DNase treatment.

5.3.3 DNase treatment

RNA isolates ore extracts are not completely free from DNA contamination, therefore DNase treatment is essential to remove traces of contaminants. For this thesis, TURBO DNA free kit (Ambion Catalogue number AM 1907) was used.

The amount of RNA required (10 µg) was calculated with each sample, with 5 µl of the 10X DNase buffer and 1 µl of DNase added to each sample. Each mix was made up to 50 µl with nuclease free water (volume individually calculated). This mixture was incubated at 37◦ C for 30 minutes followed by addition of resuspended DNase inactivation reagent (5 µl) to each sample. This was then incubated at room temperature for 5 mins before centrifuging for 10,000g for 1.5 mins. The RNA was then transferred into a fresh micro centrifuge tube. The final sample was stored at -80◦ C until needed.

5.3.4 cDNA synthesis

Total RNA (1µg) was reverse transcribed using an AMV first strand cDNA synthesis kit (New England BioLabs Inc.). Briefly the RNA was denatured for 5 minutes at 70°C to remove secondary structures which can impede long cDNA synthesis, before adding to the reaction mix containing an enzyme mix (AMV reverse transcriptase and murine RNase inhibitor) and a random primer mix (hexamer and $d(T)_{23}$ VN primers). The cDNA synthesis reaction was incubated at 25°C for 5 minutes followed by 42°C for one hour. The enzyme was inactivated at 80°C for 5 minutes. The cDNA product was then diluted to 50µL with nuclease free water and stored at -20°C until use. A 'mock' reverse transcription reaction was also carried out in which the reverse transcriptase enzyme was omitted to use as a negative (no RT) control. This was to enable the absence of genomic DNA contamination to be confirmed during later PCR steps.

5.3.5 End point PCR

The primers used for this study: Cav 1.1,Cav 1.2, Cav 1.3, Cav 3.1 and Cav 3.2 were based on those published by (Xu et al., 2003) (Table 5.1).

Amplification of cDNA was carried out in a reaction mix containing 1µl of cDNA (corresponding to 200ng), 5µl Taq polymerase (Qiagen HotStar Taq® Master Mix kit), 1µl (F + R) primer and 3µl RNase-free water, giving a total reaction volume of

10µl. End-point PCR was conducted using a Bio-Rad T100 Thermal cycler (Bio-Rad Laboratories Inc. USA). A positive control sample, no reverse-transcriptase (no-RT) and water (no template) controls were included in each PCR experiment. The PCR cycles included denaturation at 95^{o}C for 15mins, annealing for 1 min (optimised temperatures for each primer are given in Table 5.1) and extension at 72^{o}C for 1 min for 35 cycles. Using a 1% agarose gel containing SYBR safe, the PCR products were separated at 110V for one hour. The gels were imaged using the imaging software (UVP Chemi-Doc ^{TS}). Product size was estimated using a molecular weight DNA marker run simultaneously alongside the samples of interest.

Name	Primer	5'-Location (bp)	Sequence (5'-3')	Annealing temperature (°C)	Product Size (bp)
Cav 1.1	F	1233	GCGAGGTCATGGACGTGGACGA	57.6 °C	200
	R	1433	GATCACCAGCCAATAGAAGAC		
Cav 1.2	F	3527	CAGGAGGTGATGGAGAAGCCA	62.0 °C	315
	R	3842	CTGCAGGCGGAACCTGTTGTT		
Cav 1.3	F	3217	GGGGTCCAGCTGTTCAAGGGGGAA	65.9 °C	555
	R	3771	GCATGATGAGGACGAACATCATG		

Table 5. 1: PCR primer sequence for the amplification of L- and T-type calcium channels

F=forward, R = reverse, bp = base pairs

5.3.6 Literature search

On 17 December 2018, I conducted a literature search of gene expression studies in mouse myometrium using PubMed, the MEDLINE database of references and abstracts on life sciences and biomedical topics. I used the search term: "mouse myometrium gene expression" to identify all papers. The search returned 222 papers, which was also restricted to papers published within a 15 year period (from 2003), giving rise to 139 papers. The papers were manually searched further to ensure they met my stringent inclusion criteria:

(1) the study was conducted in mouse uterus

(2) the study related to gene expression (not protein) and used relevant methods including PCR, RT-PCR, qPCR

(3) full access to the article and could therefore accurately determine the reference genes used.

These criteria returned 92 papers.

Of the 92 papers retained, the method section was manually checked to determine (1) the species and gestation of mouse used; (2) the identity of the reference genes; (3) the number of reference genes used; (4) whether the authors tested a panel of genes to select the most stable; and (5) the number of candidate reference genes tested prior to selection.

The full tables of papers used and data extracted are provided in the Appendix. **Figure 5.1** also shows a flow chart of the process of paper inclusion/exclusion

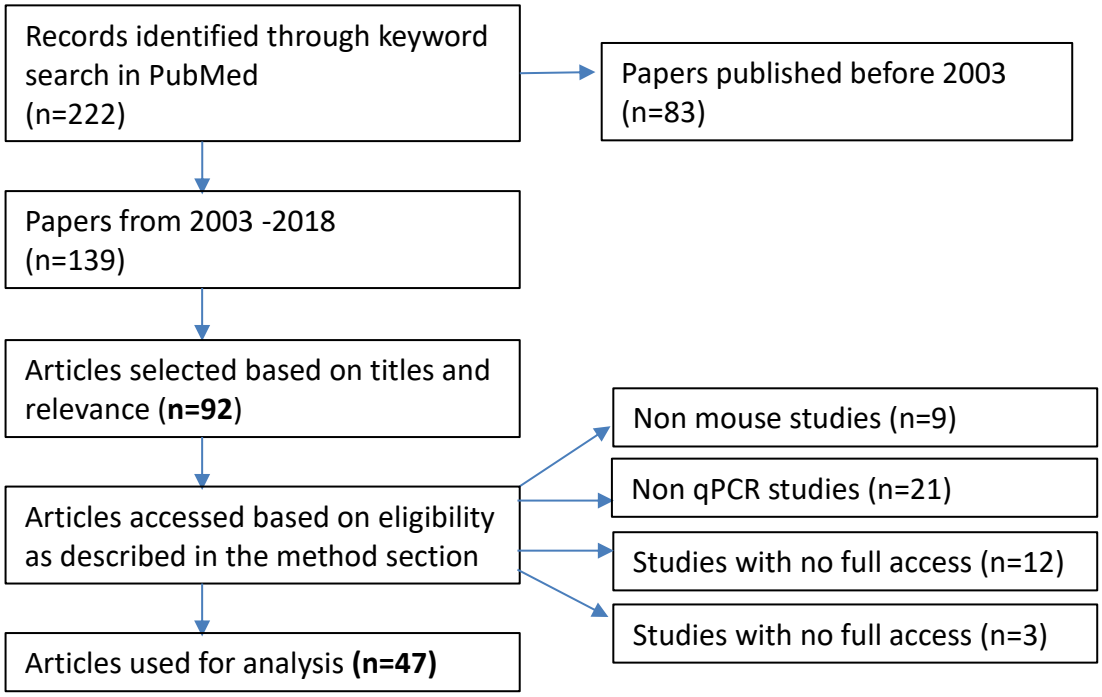


Figure 5. 1: The process of inclusion and exclusion

Flow chart shows literature search result for search words "mouse myometrium gene expression" from 2003-2018. On the right side of the chart indicates exclusion criteria and number of papers excluded based on those criteria.

5.3.7 Quantitative real- time PCR

Using qPCR, the expression levels of 12 reference genes (Table 5.2) and our genes of interest were determined across the different gestational states in mouse myometrium.

Primers for reference genes and our genes of interest were designed by and purchased from Primer Design (UK). The commercial kit contains 12 genes which have been determined from over 30000 microarray experiments to be stably expressed across different tissues, experimental conditions, different disease condition, etc. (Primer Design (2013)). The kit includes classically used housekeeping genes for comparison. Gene symbols, full name and cellular function of the reference genes are shown in Table 5.2

Using SYBR green chemistry, real-time qPCR was performed in a 10 µL reaction mix that included 5µL 2x Precision® FAST qPCR Master Mix, 0.5 µL of primer (F + R), 2 µL of nuclease free water and 2.5 µl cDNA as recommended by the manufacturer's instructions (Primer Design, UK). Reactions were carried out in triplicate on 96 well Hard Shell® PCR thin-wall plates(Bio-Rad Laboratories Inc. USA) and covered with microseal 'B' seal (Bio-Rad). For reference gene analysis plate set-up, a sample maximization strategy was used in which the number of samples per plate was maximized rather than the number of genes per plate. This was done to reduce further technical variation. Real-time PCR was conducted on a CFX Connect Real-time system (Bio-Rad Laboratories INC. USA). The Amplification protocol involved an enzyme activation step of 95^{o}C for 2mins followed by denaturation: 95°C for 5 seconds and data collection: 60^{o}C for 30 seconds for 40 cycles.

Standard curves were created for the reference genes (SDHA and ATP5B) as well as genes of interest using positive control templates for standard curves (Primer Design, UK). Standard curves were produced using serial dilutions (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1}) to check the linearity and efficiency of the PCR reactions.

5.3.8 Analysis of reference gene stability and variability

Ten samples from 10 mice from 5 gestational states were used (NP, 14d, 16d, 18d and PP). Threshold cycle (C_t) values from the samples analysed were exported to qbase^{PLUS,} which incorporates GeNorm^{PLUS} (Biogazelle) analysis program. This software was used to analyze the stability and variability of the 12 different candidate reference genes, in accordance with the software instructions. To examine the most stable reference genes, the software calculates the gene stability measure (M value) as the average pairwise variation for that gene as well as the other candidate reference genes (Vandesompele et al., 2002). Genes with the lowest M value (lower than 1.5) are considered to be the genes with most stable expression. Based on pairwise variation (Vn/n+1), the software also calculates the minimum number of genes required for RT-qPCR data normalisation between successive normalisation factors (+1, +2 and so on). As explained by Vandesompele et al., 0.15 is considered the cut-off value below which the addition of another reference gene would not be beneficial (Vandesompele et al., 2002).

5.3.9 Data analysis

To determine for gene expression changes in our genes of interest e.g. Cav 1.2 between gestational groups, data were analyzed using the methods described by Livak and Schmittgen (Livak and Schmittgen, 2001). Briefly, efficiency corrected Ct values (which take into account the differences in reaction efficiencies for the different primers) for our genes of interest in each sample group were normalized to the geometric mean of the two reference genes, SDHA and ATP5B. In doing so, this controlled for differences in sample input concentration. This normalized expression was then expressed relative to zero.

For examination of gene expression between different gestation states, we obtained an *n* of 1 resulting from a pool of 3 samples; therefore, it was not possible to determine statistical significance when comparing the expression of the gene of interest (Cav 1.2) between gestational groups.

Genes	Gene names	Cellular function
18S	Eukaryotic 18S rRNA	Ribosomal RNA
ACTB	Beta-actin	Cell migration and structure
ATP5B	ATP synthase	ATP production
B2M	Beta-2-microglobulin	Histocompatibility complex antigen class I receptor activity
EIF4A2	Eukaryotic translation initiation factor 4A2	mRNA binding to ribosome
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	signal transduction
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme
SDHA	Succinate dehydrogenase complex; subunit A; flavoprotein (Fp)	Dehydrogenase
CANX	Calnexin	Retain protein in endoplasmic reticulum
CYC1	Cytochrome c-1	Electron transport chain
UBC	Ubiquitin C	ubiquitination
RPL13A	Ribosomal protein L13A	Protein synthesis

Table 5. 2: List of the housekeeping genes and their cellular functions

5.4 Results

5.4.1 Detection of L-type calcium channels in mouse gestation

Using conventional PCR, the expression of Cav 1.1 (Figure 5.2A) was not detected in any of the myometrial samples studied. Clear expression was observed in skeletal muscle however, which was used as a positive control sample. This indicates that the PCR conditions were favourable and there were no technical issues with the reaction. As expected, Cav 1.2 was expressed at all stages of gestation and in non-pregnant tissues (Figure 5.2B). Expression of Cav 1.3 (Figure 5.2C) was not detected in any of the samples as well as in positive control. Thus Cav1.2 (α1C) is the only subunit expressed in mouse myometrium. Therefore this gene was chosen for quantitative analysis by real time qPCR.

5.4.2 Identification of published reference genes in mouse myometrium

Of the 47 papers researched, only 4 studies provided evidence that a panel of reference genes had been tested to find those that are stably expressed. Amongst all included studies, only 7 of them used a second reference gene while 4 studies used a third. The most common choice of reference genes were *GAPDH* (used in about 30% of the studies), *ACTB* (21%) and *18S rRNA* (10%) (Figure 5.3). Table 5.3 shows the number of times each gene was used in the included papers.

5.4.3 Determination of the stability of a panel of potential reference genes

The stability of 12 pre-designed candidate reference genes (Primer Design) was studied in our samples. The geNorm algorithm produced average expression stability (M) values and cycle threshold (Ct) ranges for each gene, which ranked them from lowest to highest stability (Table 5.4). In order to determine the most stable genes, the recommended Ct ranges and M values were used.

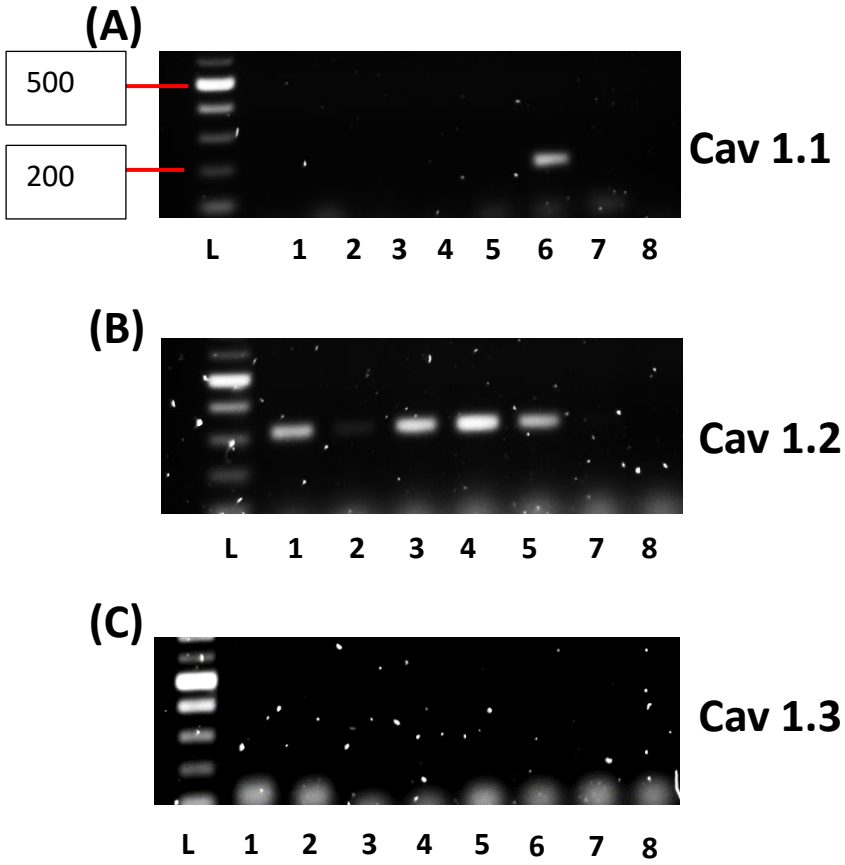


Figure 5. 2: Detection of the different L-type calcium channel (α) subunits present in mouse myometrium across different gestational states

End-point PCR was performed on samples from non-pregnant (NP), pregnant (day 14, 18 and 19) and postpartum mouse myometrium (PP).
Imaging of agarose gel after electrophoresis revealed (A) the absence of Cav 1.1 in all gestational states (B) the presence of Cav 1.2 in all gestational states, and (C) the absence of Cav 1.3 in all gestational states of mouse myometrium.
The numbers 1-8 represent non-pregnant, day 14, 16, 18, 19, and postpartum tissue, positive control, No RT and NTC respectively.
Positive controls for Cav 1.1 and Cav 1.3 are skeletal muscle and heart respectively.
NTC- no template control (NTC), no RT- no reverse transcriptase, **L**- 100 base pair ladder.

Given by ranking in all gestations studied, the most stable genes expressed were SDHA and ATP5B (**Figure 5.4**). Genes that were least stable and deemed least suitable across mouse gestation were CYC1, 18S and B2M. From literature search and as shown in Figure 5.3, 18S is commonly used as reference gene in mouse uterine samples. This result again points to the need for gene analysis to determine the most stable genes.

As shown in Figure 5.4A, the genes with the lowest M values are most stably expressed. Moreover, using geNorm, the optimal number of reference gene was estimated. Figure 5.4B shows the effect of adding an $(n+1)^{th}$ gene between two normalising factors. In my analysis, V2/3 considers the variation formed by the two most stable reference genes (ATP5B and SDHA) and added a third gene (in this case EIF4A2). With the addition of a third gene, the normalisation factor still remains lower than 0.15. Therefore, the two most stable reference genes (SDHA and ATP5B) were deemed optimal and were used in the analysis of Cav 1.2 expression in mouse myometrium across the range of gestations.

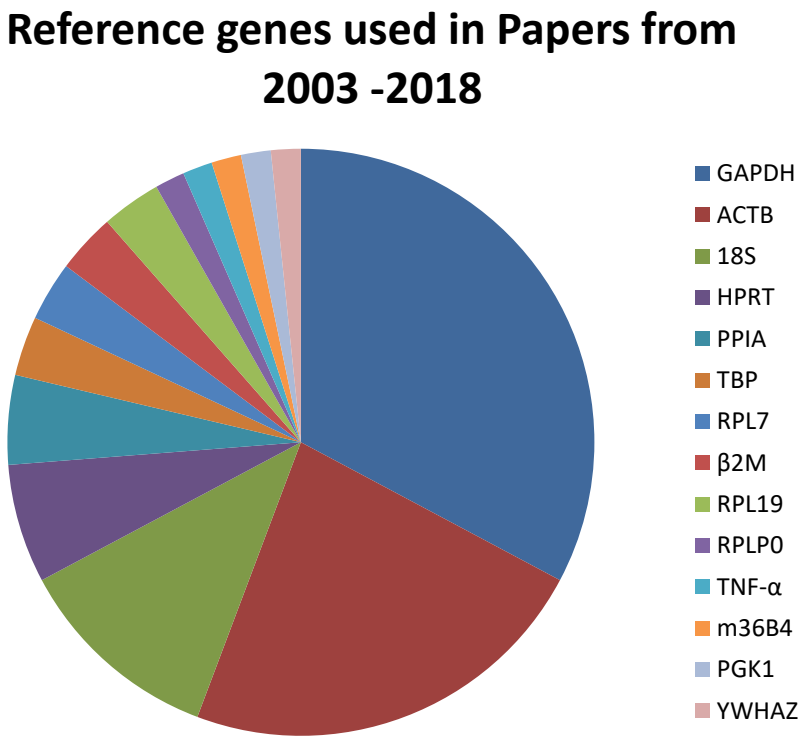


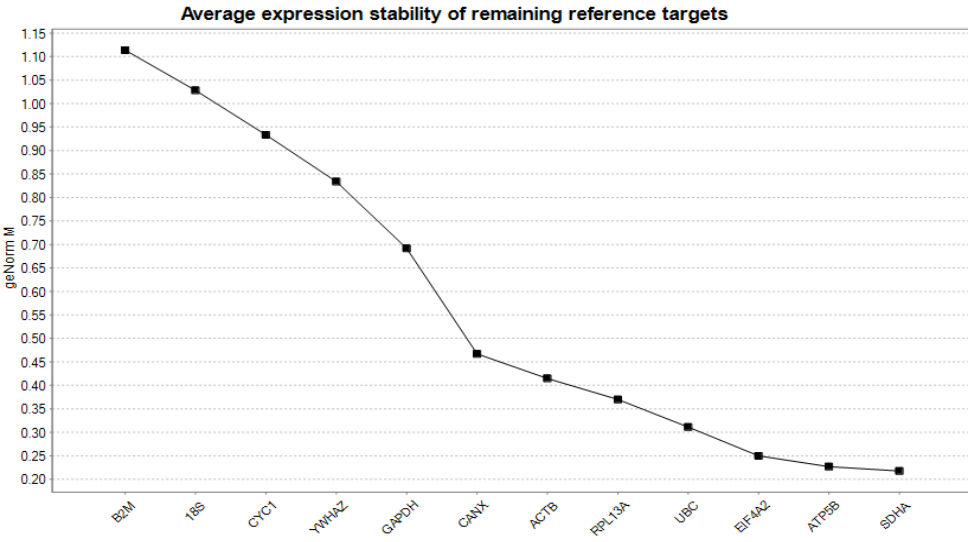
Figure 5. 3: Reference genes used in published papers from 2003 – 2018

Histogram showing the frequency reference genes commonly used in published papers. GAPDH, ACTB and 18s were the most commonly used genes within the 15 year period.

REF GENES USED	No of papers	% of total
GAPDH	20	32.78689
ACTB	14	22.95082
18S	7	11.47541
HPRT	4	6.557377
PPIA	3	4.918033
TBP	2	3.278689
RPL7	2	3.278689
β2M	2	3.278689
RPL19	2	3.278689
RPLP0	1	1.639344
TNF-α	1	1.639344
m36B4	1	1.639344
PGK1	1	1.639344
YWHAZ	1	1.639344
TOTAL	**61**	**100**

Table 5. 3: Table showing published reference genes and the frequency of use

(A)



(B)

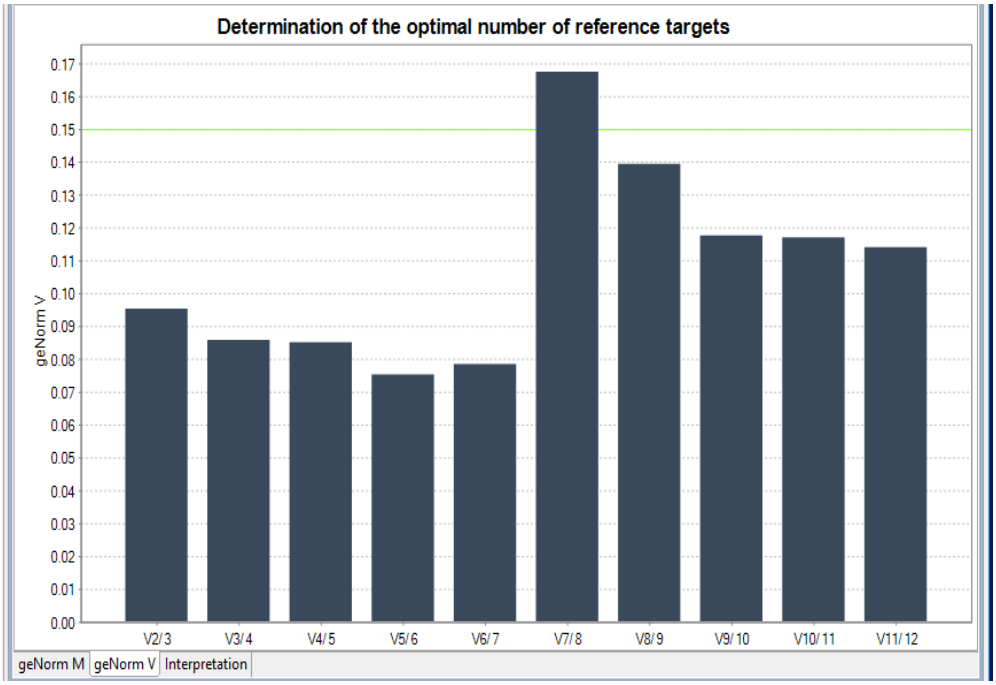


Figure 5. 4: GeNorm^{PLUS} analysis of the candidate reference genes.

(A) Average expression stability (M) and ranking of the 12 candidate housekeeping genes. The more stably expressed genes are positioned on the right side of the diagram; less stable genes are positioned on the left side. **(B)** Determination of the optimal number of housekeeping genes required for normalisation by pairwise variation (V) using GeNorm^{PLUS} software. Each bar indicates a change in the normalisation accuracy. An M value of more than 0.15 was the threshold (indicated by green line) for eliminating a gene as unstable; hence two genes would be satisfactory for normalisation.

5.4.5 Expression of Cav 1.2 at different mouse gestation

Expression of Cav 1.2 gene was determined relative to the geometric mean of the reference genes, SDHA and ATP5B.

The amplification and melt curves for each gene are represented in Figure 5.5A-F. The melt curves of all genes exhibited only one specific peak without primer dimers and non-specific amplification. The melt temperatures and amplification efficiency for Cav 1.2 were 82.0^{o}C and 93.5% respectively.

Amplification of each gene in the five samples (each sample being a pool of 3 samples) produced 15 Ct values datasets from triplicates per sample. Amplification curves for SDHA and ATP5B are shown in Figure 5.5. The standard deviations for each sample were studied and if greater than a threshold, the two replicates closest to each other were used and the third discarded from analysis.

Melt curves for the most stable genes, SDHA and ATP5B were generated (Figure 5.5B, D and F), which exhibited only one peak indicating no non-specific amplification. The melt temperatures were determined as 81.5^{o}C for SDHA and 84.0^{o}C for ATP5B. Their amplification efficiencies were 100% and 92.4% respectively.

As seen in Figure 5.6, relative expression of Cav 1.2 was lowest in day 14 pregnant tissue followed by a steady increase of expression at days 18 and 19 with maximum expression in postpartum tissues. Interestingly, non-pregnant samples also had high levels of expression.

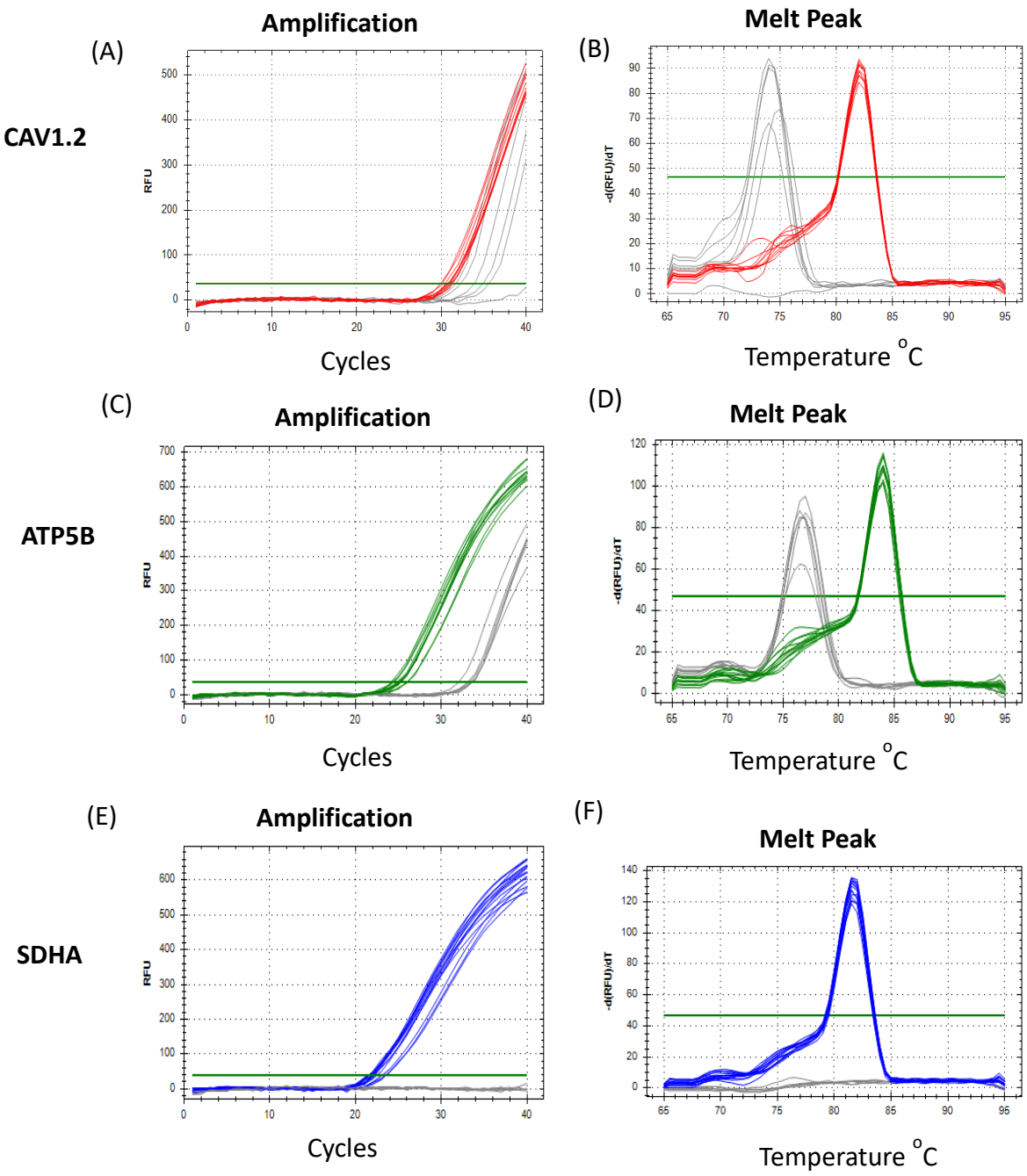


Figure 5. 5: Amplification and melt curves for Cav 1.2, SDHA and ATP5B.

(**A, C and E**) show amplification curves for Cav 1.2, SDHA and ATP5B, with efficiency of 93.5% , 100% and 92.4% respectively.; (**B,D and F**) show the melt peak of SDHA and ATP5B, with melting temperatures of 82 °C , 81.5°C and 84.0°C respectively.

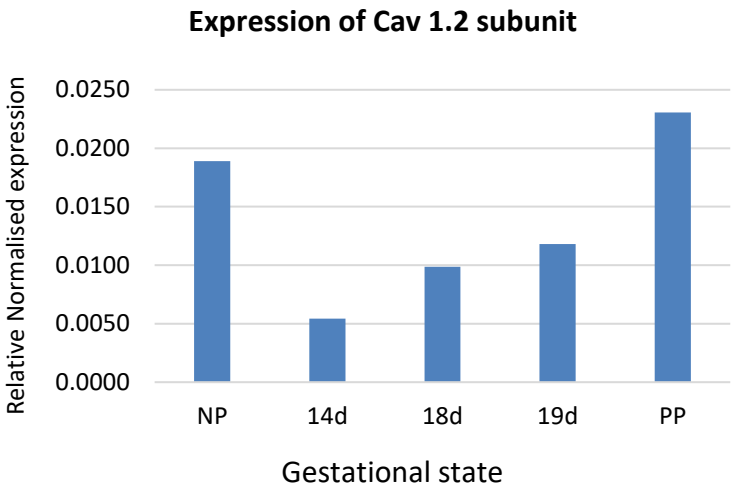


Figure 5. 6: Comparison of the expression of Cav 1.2 subunit across different gestational states in mouse myometrium, determined by qPCR.

The expression of Cav 1.2 in each sample group was normalised to the expression of the two reference genes, SDHA and ATP5B. Taking non-pregnant (NP) as control, the expression of Cav 1.2 drops at day 14, followed by steady increase towards term (day 19) and further increases postpartum (PP). Postpartum tissue was collected close to delivery.

5.5 Discussion

The relationship between changes in intracellular calcium concentration and myometrial contraction is well documented (Longbottom et al., 2000, Word et al., 1993, Fomin et al., 2009). It is the L-type calcium channel which plays a major role in increasing intracellular calcium during excitation-contraction coupling. Despite the importance of the L-type calcium channels especially the α1C subunit in uterine contractility, only a few studies have extensively examined the levels of expression throughout gestation and post labour. To the best of my knowledge, no study has investigated its expression throughout gestation in the mouse.

This chapter was undertaken to determine the expression and relative abundance of different calcium channels throughout gestation in mouse myometrium. In particular, it describes the changes in expression of L-type calcium channel α subunits throughout pregnancy and after labour. We also determine the most stable reference genes for use in studies of gene expression in mouse myometrium at different gestational states as this was previously unreported.

My results show the absence of Cav 1.1 and Cav 1.3 subunits in mouse uterine tissues at any gestational time point. Although it may be difficult to conclude for the Cav 1.3 subunit, especially since it was also not detected in the positive control (heart). As expected, Cav 1.2 was detected in uterine tissues in all pregnant samples as well as non-pregnant and postpartum tissues.

Published stable reference genes versus geNorm analysis

Real-time qPCR was carried out to quantify the expression of the Cav 1.2 in the different uterine samples. This method has become very popular in detection and measurement of gene expression. Due to the sensitivity of gene expression, the use of reference genes is used as the gold standard for efficient normalization (Almeida et al., 2014). It is known that the choice of stable and suitable reference gene is essential for accurate and reproducible gene expression analysis in real-time PCR experiments (Xu et al., 2016), hence it was important that I determine the most stable genes for use in this study.

In this study, 12 housekeeping genes were investigated; 18S, ACTB, ATP5B, B2M, CANX, CYC1, EIF4A2, GAPDH, RPL13A, SDHA, UBC, and YWHAZ. These genes consisted of genes known to be stably expressed in a number of tissues and experimental conditions and included some previously reported as suitable reference genes in studies of myometrial gene expression. Our literature search revealed the most commonly used genes in mouse myometrium studies to be GAPDH, 18S and ACTB. Only 4 used geNorm software to determine the most suitable reference genes. Interestingly, in this study, these genes commonly used as reference genes in mouse myometrium were ranked amongst the most variable genes by geNorm across gestational states in the mouse uterus. This highlights the need to determine the most appropriate reference genes for every protocol and tissue under investigation.

It is recommended that gene expression data may be more reliable if it is normalised by geometric means of more than one reference gene (Bionaz and Loor, 2007). In our samples, pairwise variation analysis showed that the optimal number of reference targets for qPCR data normalisation is two, indicating the most suitable reference genes with little variation across mouse gestational sample subsets to be ATP5B and SDHA. This also indicates that their geometric means would be sufficient and provide accurate data normalization factor for expression across gestational states of mouse uterine tissues, which I have applied in this study.

Quantification of Cav 1.2 expression in mouse uterine tissues

Using ATP5B and SDHA as reference genes and non-pregnant samples as a control, our results indicate a marked decrease in expression at day 14 (compared to Non-pregnant). This was followed by an increase at days 18 and 19, although levels did not reach that of non-pregnant samples. A further increase in expression was also noted in postpartum samples. Our results are somewhat similar to the findings of Mershon et al who reported mRNA expression of Cav 1.2 (α1C subunit) in rat myometrium (Mershon et al., 1994). Using dot blot analysis, they showed that compared to non-pregnant samples, expression at day 7 remained unchanged. This was followed by a significant decrease at day 14. The expression then increased

steadily in the last three days of gestation, returning to control levels on day 21. They also showed marked reduction of expression during labour followed by a sharp increase in postpartum samples (Mershon et al., 1994). Similar results by Tezuka et al using RT-PCR, showed a gradual increase in Cav 1.2 mRNA expression before labour, however this was followed by a decrease during labour and postpartum (Tezuka et al., 1995). Despite the variations in results, our findings support the generalized trend of increased mRNA expression of Cav 1.2 as gestation progresses toward term.

As described by Mershon et al, the state of quiescence at mid-gestation (day 14) may explain the reduced expression noticed at day 14. The onward increase in the expression of Cav 1.2 towards term may indicate the role of L-type calcium channels in the myometrium in preparation for parturition (Mershon et al., 1994).

In summary, this chapter indicates the absence of Cav 1.1 and Cav 1.3 subunits across mouse gestational uterine samples, but shows the presence of Cav 1.2 in all gestational states studied. Overall, the end-point PCR data showed some primer dimer formation, which could be explained by lack of proper optimization of the conditions used. Nevertheless, this didn't affect my findings as my main focus was detecting the presence or absence of the α1 subunits in these samples.

This chapter also enforces the need to determine the most suitable reference genes especially when involving different experimental procedures and on different tissue types. My data provides fresh insight into the expression of L-type calcium channel (Cav 1.2) at different gestational states in mice. Overall, increased expression was noticed in pregnancy towards term supporting the role of L-type calcium channel in parturition process. I have also identified the most suitable reference genes for studies of gene expression in mouse uterus using pan-gestational tissues which will be a useful addition to the literature and valuable tool for others to use in future studies.

5.5.1 Limitations of this study

I was unable to investigate the expression of the L-type calcium channel subunit, Cav 1.2 in labouring mouse uterine samples. It would be interesting to see the level

of expression between term-pregnant and postpartum in the mouse uterus as this may have provided further insight to the events and influence of L-type calcium channels during labour.

Further optimisation of the end-point PCR technique is required e.g. to limit primer dimer formation and hence increase efficiency of the PCR reaction.

I was unable to investigate the presence of Cav 1.4 subunit in my samples.

There was lack of control for Cav 1.2 as well as positive controls not detected in Cav 1.3, Cav 3.1 and Cav 3.2. Further studies would explore suitable positive controls in mouse and optimise the conditions in which PCR was undertaken.

The samples from similar gestation were pooled, making it impossible to calculate the standard error mean and determine significance levels of the effect seen between gestations.

5.6 Conclusion

This study is the first to quantify the expression of L-type calcium channel subunit (Cav 1.2) across mouse gestational states.

In this chapter, I have shown that Cav 1.1 and Cav 1.3 are not present in mouse myometrial tissues, but Cav 1.2 was detected across all gestational stages including non-pregnant and postpartum tissues.

I also carried out literature search of the most commonly used reference genes. The result showed GAPDH, ACTB and 18S to be the most commonly used reference genes and the choice of these genes was not predetermined by the use of gene analysis software.

Using geNorm software I investigated further the most stable genes throughout mouse gestational states. My results showed ATP5B and SDHA to be the most stable expressed genes while GAPDH and ACTB were among the least stable genes.

Finally, using ATP5B and SDHA as reference genes, the expression of Cav 1.2 subunit was determined. Results showed an expression in pregnancy which increased towards term, although the levels did not reach that of non-pregnant. The highest expression was seen immediately after delivery.

Chapter 6

Final Discussion

Chapter 6

Final Discussion

Clinically, the use of magnesium as a tocolytic has been questioned. Nevertheless, in the UK, it has been recommended for use in very preterm cases- not as a tocolytic but for neuroprotection. The main aim of this study therefore was to investigate how combining magnesium with other commonly used tocolytics might improve tocolytic efficacy and how physiological and hormonal factors may affect its efficacy. In doing so, the effect of magnesium at different gestational states was studied. Since magnesium is known to act on the L-type calcium channels, the mRNA expression of α1C subunit in mouse was also determined. I believe this work gives a better insight into why some tocolytics may not be effective clinically.

6.1 Magnesium and Gestation

There has only been one study that has investigated the effect of magnesium in mid-pregnant mouse myometrium; this study was carried out in a liposaccharide mouse model (Sugawara et al., 2007).This is the first study however that has studied magnesium's effect across mouse gestational states of spontaneous and oxytocin-induced contractions. In both spontaneous and oxytocin-induced contractions, magnesium reduced contractions, in a concentration-dependent manner.

My results highlight magnesium's relaxing effect on uterine smooth muscle. This effect is due to magnesium's cationic competition with calcium (Phillippe, 1998), producing a fall in $[Ca^{2+}]i$ and calcium levels within the SR. At all gestational states, the addition of $MgSO_4$ triggered a decrease in frequency before a fall in the force of contractions. This finding suggests that magnesium may have a role in intracellular coupling, decreasing gap junctions (Rimkute et al., 2018), thereby reducing the possibility of frequent and synchronised contractions. These results also showed that magnesium's effect on spontaneous contractions was more potent at term and

least potent in non-pregnant tissues. Magnesium is thought to act by decreasing calcium entry and excitability. This mechanism may not explain the effect seen in our samples except the increased α-1 expression gives rise to increased response to magnesium blockade. My data suggests that at term, magnesium's effect is sufficient to reduce excitable, and make action potential firing less likely.

To investigate the effect of magnesium in the presence of an agonist, its effect on oxytocin-induced contractions was determined. My data showed that a greater concentration of magnesium was needed to inhibit oxytocin-induced contractions compared to spontaneous in all pregnant gestations studied. There was however no significant change between non-pregnant groups. In general, there was no significant difference in magnesium's effect on oxytocin-induced contractions across gestations. Oxytocin's mechanism of increasing membrane potential and releasing calcium from the SR, will counteract magnesium effect, and may partly explain why its effect is minimal.

My data shows that the preterm uterus is less sensitive to the tocolytic effect of magnesium than term uterus, which could explain the lack of efficacy seen in the clinics. In the presence of oxytocin, its efficacy was further reduced at all gestations studied, with oxytocin decreasing the potency of magnesium. Therefore, magnesium's actions in mouse are influenced by both gestational state and hormones, with magnesium been less effective in early gestation with oxytocin present and most effective at term in the absence of oxytocin.

A limitation of this study is the use of $MgSO_4$ at supra-pharmacological doses in the *in vitro* model. The appropriate total dosage for magnesium as a foetal neuroprotectant is not known, nevertheless, it is suggested that 6g is used over a 30 minute period followed by 2g continuous infusion per hour (Rouse et al., 2009) or 4g for 30 minutes followed by 1g per hour until birth (Magee et al., 2011). In preeclampsia, the upper limit of the therapeutic plasma level for seizure management is 3 mM, with toxicity occurring at concentrations > 3.5 mM. There is respiratory paralysis at 5-6 mM, cardiac conduction defects at > 7.5 mM, and cardiac arrest at > 12.5 mM (Lu and Nightingale, 2000, Tang et al., 2015). In my

experiments, the rationale for using high concentration of $MgSO_4$ was to provoke a response that could potentially occur at the upper end of its use. In normal clinical practice, where infusion rates are generally set at 1 or 2 g/hr after a bolus of 6g or 4g, it is rare to observe levels > 4 mM, nevertheless, with the organ bath method, higher concentrations are needed to produce similar effects to what is seen *in vivo*. It cannot also be assumed that the contractile changes shown *in vitro* would also be seen *in vivo* as the multitude of oxytocic effects *in vivo* could imply the need for further studies.

6.2 Effect of Individual Tocolytics.

The effect of indomethacin, atosiban and nifedipine has individually been investigated *in vitro* and *in vivo* and well-documented in term pregnant human myometrium. It was however important to establish their effect on pregnant mouse myometrium. The result will provide a basis on interpreting the data for the dual tocolytics work in this thesis.

In my tissues, indomethacin concentration-dependently inhibited spontaneous contractions, however, its effect were less in oxytocin-induced contractions. At higher concentrations, indomethacin abolished contractions rapidly indicating that it may be acting via a mechanism other than inhibiting COX and prostaglandin production. Prostaglandins are produced by COX activity throughout pregnancy with their levels increasing steadily through gestation and peaking before delivery, stimulating myometrial activity. Although it is well known that indomethacin inhibits prostaglandin synthesis, indomethacin is also believed to function via prostaglandin-independent mechanisms, such as inhibition of nitric oxide synthase and phosphodiesterase 4 (Garza et al., 2004). Perhaps in-depth study of the other mechanisms may be needed to have a better understanding of indomethacin's activity on myometrial contractions. Atosiban also had a concentration-dependent inhibitory effect on force and frequency of oxytocin-induced contractions. This effect is as a result of atosiban's effect on oxytocin and vasopressin receptors. While

investigating the effect of nifedipine on uterine contractions, I found that nifedipine produced a time-dependent effect on contractions. Nifedipine inhibited contractions in both spontaneous and oxytocin-induced contractions. Nifedipine is classed as a dihydropyridine and also acts by binding to the VGCCs, inhibiting action potential and uterine contractions. Nifedipine causes a reduction in $[Ca^{2+}]i$ levels by blocking the extracellular flow of calcium into the myometrial cells thereby decreasing contractions (Moynihan et al., 2008a).

All the tocolytics examined operate via different intracellular pathways and have different side effects

6.3 Effect of Combining Magnesium and Other Tocolytics

Since the effect of magnesium on myometrium has been controversial, I first established the effect of magnesium in my tissues (Chapter 3). I then determined the inhibitory effect and IC_{50} values of the other tocolytics (indomethacin, atosiban and nifedipine), before proceeding to investigate its effect *in vitro* in combination with other tocolytics. It was hypothesised that combining two tocolytics that act on different contractility pathway may produce a synergistic inhibition. The results of the hypothesis are presented in Chapter 4 of this thesis.

The results showed that combining magnesium and indomethacin produced a concentration-dependent reduction of spontaneous contractions, which was additive. In oxytocin-induced contractions however, the effect of the combination was antagonistic. This antagonistic effect may be as a result of indomethacin not been able to antagonise oxytocin's action. Similar conclusion was drawn from another study that investigated the effect of indomethacin on rat uteri (Vane and Williams, 1973). The combination of magnesium and atosiban produced a synergistic concentration-dependent inhibitory effect on oxytocin-induced contractions. The combination abolished contractions at even low concentration. This finding is similar to that obtained in human myometrium (Arrowsmith et al., 2016). Although a singular concentration of magnesium and nifedipine caused a

significant inhibitory effect compared to control, this did not reach statistical significance when compared to either drug alone. This might be as a result of magnesium reducing calcium entry via the L-type calcium channel and nifedipine blocking L-type calcium channels: similar mechanisms.

Most tocolytics are associated with adverse effects on the mother and on the foetus *e.g* β-mimetics, indomethacin and nifedipine (Goldenberg, 2002, Nelson et al., 2014, van Veen et al., 2005, Khan et al., 2010) Nelson et al., 2014, Khan K et al 2010, van Veen et al 2005), while others like atosiban have been shown to have no evident effect on prolongation of pregnancy (Romero et al., 2000). Studies show that although these drugs may be capable of providing some clinical benefit of delay, the high doses required to achieve relaxation of the myometrium increases the risk of adverse systemic effects (van Veen et al., 2005, Khan et al., 2010). Indomethacin for example is associated with an increased risk for severe intraventricular haemorrhage, and necrotising enterocolitis (Sawdy et al., 2003) .

One limitation of this study is that samples were obtained from term pregnant and not labouring mice, whereas tocolytics are used in preterm labouring women. These results therefore may not translate to clinical scenarios. The strength of this study however, is the combination of magnesium, a neuroprotectant, with the most commonly used tocolytics in the UK clinical practice. The clinical relevance of my findings suggests that combining two tocolytics acting via different pathways may be more effective and may also result in low doses being used, reducing side effects and improving perinatal outcome. To confirm the clinical effectiveness of the dual combination of tocolytics with $MgSO_4$, and demonstrate the synergistic effect of $MgSO_4$ + atosiban, clinical studies are required.

Comparing all combinations in the presence of oxytocin, my data indicate that magnesium and atosiban are more potent than magnesium and nifedipine which in turn are more potent by magnesium and indomethacin. We therefore postulate that drugs acting via different mechanistic pathways may produce a synergistic effect. The clinical implications are that using magnesium as a foetal

neuroprotectant may enable existing tocolytics especially those that function at different intracellular sites to be administered with improved efficacy and improved safety. This may reduce the quantity of drug required to achieve therapeutic efficacy, reduce the likelihood of maternal and foetal side effects, and may therefore represent a significant advancement for maternal-foetal medicine.

6.4 Effect of Acidification on Magnesium and Atosiban

The effect of acidification on pregnant human myometrium has previously been documented with changes in extracellular and intracellular pH altering uterine contractions (Parratt et al., 1995, Taggart and Wray, 1993). Since my data suggests that the effect of magnesium is improved when combined with atosiban, it was important to investigate how acidification may affect its tocolytic efficacy *in vitro*. My data showed that acidic external pH reduces the tocolytic efficacy of magnesium combined with atosiban by increasing the force and frequency of uterine contractions. This may partly be as a result of H^+ and Ca^{2+} competing for intracellular binding sites (Wray, 1988) leading to increase in levels of intracellular free Ca^{2+}.

It is known that uterine perfusion reduces during labour contraction as the blood vessels are temporarily occluded. This occlusion results in hypoxia/ ischaemia and acidification (Harrison et al., 1994). The work reported here suggests that acidification occurring during forceful contractions of labour may result in a more forceful and frequent uterine contractions. If similar changes occur in preterm labour, the potency of tocolytics may be affected. To explore this further, the effect of intracellular acidification in the presence of tocolysis should be investigated.

6.5 L-type Calcium Channel Expression in Mouse Uterus

In Chapter 3, I showed that there were variations in the efficacy of magnesium at different gestational states. Since magnesium is thought to act at the L-type calcium channel, it was therefore important to determine the expression of L-type calcium

channels across different gestational states of mice. Understanding this would give better insight to the use of tocolytics (especially those acting on L-type calcium channels) in preterm labour. I therefore examined the mRNA expression and relative abundance of different calcium subunits throughout gestation. Using conventional PCR technique, I found no expression of Cav 1.1 in myometrial samples studied. As expected, Cav 1.2 was expressed in all gestations including non-pregnant and post-partum.

In order to quantify the level of Cav 1.2 gene expression, I first determined the most stable reference genes at different gestational states in mouse myometrium. Results from literature search suggest GAPDH, 18S and ACTB as the most frequently used housekeeping genes in mouse myometrium. It was also noted that most of the studies did not employ the use of any gene expression software. Therefore to confirm the stability of these housekeeping genes, we used geNorm to investigate 12 genes. Results showed these genes to be among the least stable across different gestations. This finding enforces the need to determine the most suitable reference genes especially when there is variation in experimental procedures. Pairwise variation analysis showed the optimal number of reference targets to be two and indicated ATP5B and SDHA as the most stable housekeeping genes. The geometric means were then used as data normalisation factor in Cav 1.2 expression across gestations in mouse uterus.

Compared to non-pregnant (control) the qPCR data indicate a marked decrease in expression at day 14, which was followed by an increase at days 18 and 19 although levels did not reach that of non-pregnant. It is suggested that the state of quiescence at mid-gestation (day 14) may explain the reduced expression noticed at day 14 while the onward increase in expression towards term may indicate the role of L-type calcium channels in preparation for parturition. Interestingly, a sharp increase was also seen in postpartum samples, with expression greater than all gestations studied.

In summary, this study has demonstrated significant changes in the expression of the L-type VGCC in pregnancy. An increase in the expression of calcium channels,

also indicating an increase in dihydropyridine binding sites, was seen from day 14 through the last day of gestation. This supports a role for the L-type calcium channels in uterine smooth muscle contraction. With no drastic increase in expression at term, it implies that the L-type channels may not be important in triggering the onset of labour. My data suggest that at term, magnesium is able to reduce excitability and reduce the firing of action potential. However, it may be difficult to explain gestational differences by the effect of calcium entry, except the increased expression of the α1C subunit results in increased reception to magnesium blockade. Further studies are necessary to correlate the results of mRNA quantification to functional data.

6.6 Summary of Clinical Implications

Management of preterm labour is very complex as different aspects have to be considered including the impact on the unborn baby, and complications on both mother and baby. The goal of managing preterm labour is to prolong pregnancy as safely as possible and enable foetal development and maturation. The current tocolytics in clinical practice delay labour long enough until corticosteroids are administered and until maternal transfer to a hospital with appropriate neonatal facilities.

In summary, my results showed an additive effect with magnesium and nifedipine, which may be due to a combined effect on calcium ion influx. Most women with severe preeclampsia and may be given $MgSO_4$ (Magee et al., 2003). A trial involving 1469 women, given both $MgSO_4$ and nifedipine (Magee et al., 2005), showed that there was no documented case of neuromuscular blockade, and there was no excess of maternal hypotension. They also calculated the risk of neuromuscular blockade among the women to be <2%. They therefore suggested that such a risk would not justify recommendations to avoid concomitant therapy with both drug, especially given nifedipine's ease of administration and effectiveness for the treatment of severe preeclampsia (Magee et al., 2005). On the other hand, several studies have shown that the combination of magnesium and nifedipine may

potentiate the side effects of the drugs such as myocardial depression and hypotension (Thorp et al., 1990, Neustein et al., 1998). Although it may be difficult to extrapolate clinical significance from animal studies, however, if the effect of both drugs *in vivo* results in significant induction of hypotension, this may jeopardize uterine perfusion pressure and subsequently lead to foetal distress in pregnant patients (Neustein et al., 1998). We also have to consider that the side effects common to both drugs (such as cardiovascular side effects) may also be enhanced by this combination.

My results also show that there is a synergistic tocolytic effect when magnesium is combined with atosiban. This synergistic effect exhibited by the combination with magnesium would result in the use of lower effective concentrations of atosiban, hence leading to a reduction in maternal and foetal side effects. Despite the tocolytic efficacy of both drugs individually, the clinical use of atosiban remains limited by its high cost (Doret et al., 2003). This suggests that the combination of magnesium with atosiban may also have an added advantage of decreasing the cost of tocolysis. These drugs used individually are not without their side effects, and my work may lead to less drugs being used therefore less side effects. A Cochrane review showed that about 38% of women treated with antenatal magnesium report any adverse effect (Bain et al., 2013). 50% of these patients have an increased risk of respiratory depression, and other side effects include neuromuscular blockade, postpartum haemorrhage, urinary stone formation and hypotension. Atosiban has also been shown to be associated with tachycardia, nausea, vomiting, headaches, dizziness and hyperglycaemia. Therefore, the synergistic tocolytic effect of magnesium used in combination with atosiban may provide the opportunity to improve the maternal and foetal side effect profile.

My results also showed that the synergistic tocolytic effect was reversed by extracellular acidification. If the increase in force and frequency of contractions seen with acidification is characteristic of preterm labour, full knowledge of the mechanism is of value to clinicians as more caution would be taken when administering this combination.

6.7 Future work

From the findings in this thesis, there are potential opportunities for further research that could be pursued.

Magnesium

The finding that magnesium reduced contractions in pregnant and non-pregnant mouse *in vitro* could be further explored by investigating magnesium's effect on a preterm labour mouse model *in vivo*. Also, it will be interesting to investigate the effect of magnesium on intracellular calcium signalling by using calcium sensitive indicator Indo-1 AM.

Potassium channels reduce membrane excitability and its expression is reported to decrease significantly in late pregnancy compared to non-pregnant and early pregnant stages (Khan et al., 2001, Mazzone et al., 2002). Further studies could explore the possible effect of magnesium on potassium channels at different gestations.

Combined tocolytics

This *in vitro* study was carried out to determine the tocolytic efficacy of combination therapy involving magnesium, however, only biochemical interaction between the drugs was achieved with this *in vitro* mouse model. *In vivo* studies will be beneficial and would give better insight into pharmacodynamics and pharmacokinetic interaction dependent on drug absorption and metabolism. It is also important to evaluate the foetal side effect of combined tocolytics on animal models before carrying out any human studies. Similar to the effect of magnesium on contractions, the effect of combining tocolytics could be explored further by measuring force and calcium signalling simultaneously. In addition, the combined tocolytic effect could be investigated on preterm labouring instead of term non-labouring tissues. Once the combined tocolytic effect is proven in human labouring

tissues, and foetal side effects of combination are evaluated, further clinical studies would be needed.

The finding that extracellular acidification reversed the tocolytic efficacy of magnesium + atosiban could be investigated further by examining how intracellular pH is affected, using the pH-sensitive indicator carboxyl-SNARF. In addition, the effects of other factors like lactate, stretch and hypoxia could also be investigated.

Calcium channel expression

This thesis has shown the mRNA expression in non-pregnant, days 14, 18, 19 and postpartum tissues. It will be interesting to investigate the mRNA expression between non-pregnant and day 14 as well as labouring tissues. Although quantifying mRNA expression is a good indicator of gene regulation, is often an excellent indication of the presence of protein. Nevertheless, relative mRNA levels may or may not be directly correlated to the relative abundance of proteins (Vogel and Marcotte, 2012); hence, protein expression of the α1C subunit should also be investigated. This could be done using western blot technique.

In this thesis, whole uterine tissue has been used. Further studies should compare the expression between myometrium and whole or full thickness tissues. Studies have also shown variation between longitudinal and circular muscles of rat uterus (Lee et al., 2009, Ohkubo et al., 2005); therefore investigating the difference in mouse myometrium might prove useful.

6.8 Conclusion

The main findings of this thesis are:

- Magnesium concentration-dependently inhibits spontaneous and oxytocin-induced uterine contractions of both pregnant and non-pregnant mouse, with its greatest effect occurring in term-pregnant

- The combination magnesium plus atosiban produced a synergistic effect and was more potent than magnesium plus nifedipine and magnesium + indomethacin.
- Extracellular acidification reversed the tocolytic efficacy of magnesium plus atosiban, causing increase in force and frequency of contractions.
- There is increased expression of the α1C subunit during pregnancy and progresses towards term supporting the role of L-type calcium channel in parturition process.

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Appendix 1

Publications Arising From This Thesis

Publications Arising From this Thesis

Journal article

OSAGHAE, B., ARROWSMITH, S., WRAY, S. (Submitted). Gestational and hormonal effects on magnesium sulfate's ability to inhibit mouse uterine contractility. *Reproductive Sciences*

Abstract and conference proceedings

OSAGHAE, B., ARROWSMITH, S., WRAY, S. (2016). The dual tocolytic effect of magnesium plus nifedipine on mouse myometrial contractility. *Preterm Birth Research Conference.* Leeds, United Kingdom

OSAGHAE, B., ARROWSMITH, S., WRAY, S. (2016). The effect of dual combination of $MgSO_4$ with atosiban and indomethacin on myometrial contractions of pregnant mouse. *Preterm Birth Research Conference.* London, United Kingdom

OSAGHAE, B., ARROWSMITH, S., WRAY, S. (2016). The effect of dual combination of $MgSO_4$ with atosiban and indomethacin on myometrial contractions of pregnant mouse. *Proceedings of the Physiological Society* 37, PCA33. Dublin, Ireland.

OSAGHAE, B., ARROWSMITH, S., WRAY, S. (2015). Magnesium sulphate inhibits spontaneous and oxytocin induced contractions of mouse myometrium. *Proceedings of the European Young Physiologist Symposium*. Kaunas, Lithuania

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OSAGHAE, B., ARROWSMITH, S., WRAY, S. (2015). $MgSO_4$ in combination with other tocolytics: a promising treatment for threatened preterm birth. *Inaugural Preterm Birth Research Conference.* Liverpool, United Kingdom.

Appendix 2

List of Papers Searched For Reference Gene Analysis

List of Papers searched For Reference Gene Analysis

Titles	Year Published
Upregulation of HB-EGF, Msx.1, and miRNA Let-7a by administration of calcitonin through mTOR and ERK1/2 pathways during a window of implantation in mice.	2018
IRF5 is increased in labouring myometrium and regulates pro-labour mediators.	2018
Generation of Mouse for Conditional Expression of Forkhead Box A2.	2018
Uterine Foxl2 regulates the adherence of the Trophectoderm cells to the endometrial epithelium.	2018
Seipin deficiency leads to defective parturition in mice.	2017
Expression of Matrix Metalloproteinases in the Mouse Uterus and Human Myometrium During Pregnancy, Labor, and Preterm Labor.	2018
Selenoprotein N Was Required for the Regulation of Selenium on the Uterine Smooth Muscle Contraction in Mice.	2017
The transcriptional repressor GATAD2B mediates progesterone receptor suppression of myometrial contractile gene expression.	2017
Bitter taste receptors as targets for tocolytics in preterm labor therapy.	2017

Distinct effects of short- and long-term type 1 diabetes to the placental extracellular matrix and fetal development in mice.	2017
The Local and Systemic Immune Response to Intrauterine LPS in the Prepartum Mouse.	2016
Lactate produced during labor modulates uterine inflammation via GPR81 (HCA₁).	2017
Modeling hormonal and inflammatory contributions to preterm and term labor using uterine temporal transcriptomics.	2016
Age-related changes in murine myometrial transcript profile are mediated by exposure to the female sex hormones.	2016
The transient receptor potential vanilloid 4 channel modulates uterine tone during pregnancy.	2015
Constitutive activation of transforming growth factor Beta receptor 1 in the mouse uterus impairs uterine morphology and function.	2015
Prolonged pregnancy in women is associated with attenuated myometrial expression of progesterone receptor co-regulator Krüppel-like Factor 9.	2015
FSH receptor (FSHR) expression in human extragonadal reproductive tissues and the developing placenta, and	2014

the impact of its deletion on pregnancy in mice.	
The inwardly rectifying K+ channel KIR7.1 controls uterine excitability throughout pregnancy.	2014
Inhibition of infection-mediated preterm birth by administration of broad spectrum chemokine inhibitor in mice.	2014
Lactoferrin-iCre: a new mouse line to study uterine epithelial gene function.	2014
Follistatin is essential for normal postnatal development and function of mouse oviduct and uterus.	2015
Statins prevent cervical remodeling, myometrial contractions and preterm labor through a mechanism that involves hemoxygenase-1 and complement inhibition.	2014
TGFBR1 is required for mouse myometrial development.	2013
Expression of the small conductance Ca^{2+}-activated potassium channel subtype 3 (SK3) in rat uterus after stimulation with 17β-estradiol.	2014
Increased tissue levels of omega-3 polyunsaturated fatty acids prevents pathological preterm birth.	2013
Uterine endoplasmic reticulum stress and its unfolded protein response may regulate caspase 3 activation in the pregnant mouse uterus.	2013

Distinct spatiotemporal expression of serine proteases Prss23 and Prss35 in periimplantation mouse uterus and dispensable function of Prss35 in fertility.	2013
Myometrial immune cells contribute to term parturition, preterm labour and post-partum involution in mice.	2012
Mice deficient in surfactant protein A (SP-A) and SP-D or in TLR2 manifest delayed parturition and decreased expression of inflammatory and contractile genes.	2013
The microRNA (miR)-199a/214 cluster mediates opposing effects of progesterone and estrogen on uterine contractility during pregnancy and labor.	2012
Stromal-to-epithelial transition during postpartum endometrial regeneration.	2012
Generation of myometrium-specific Bmal1 knockout mice for parturition analysis.	2012
Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface.	2012
N-acetylcysteine prevents preterm birth by attenuating the LPS-induced expression of contractile associated proteins in an animal model.	2012
MicroRNA-200a serves a key role in the decline of progesterone receptor function leading to term and preterm labor.	2012

Smoothelin-like 1 protein is a bifunctional regulator of the progesterone receptor during pregnancy.	2011
Mast cells reside in myometrium and cervix, but are dispensable in mice for successful pregnancy and labor.	2012
How does progesterone relax the uterus in pregnancy?	2011
miR-200 family and targets, ZEB1 and ZEB2, modulate uterine quiescence and contractility during pregnancy and labor.	2010
Decidual PTEN expression is required for trophoblast invasion in the mouse.	2010
Neuromedin B and its receptor induce labor onset and are associated with the RELA (NFKB P65)/IL6 pathway in pregnant mice.	2011
Decreased expression of the rat myometrial relaxin receptor (RXFP1) in late pregnancy is partially mediated by the presence of the conceptus.	2010
SK3 channel expression during pregnancy is regulated through estrogen and Sp factor-mediated transcriptional control of the KCNN3 gene.	2010
Broad tissue expression of membrane progesterone receptor Alpha in normal mice.	2010
The contribution of Kv7 channels to pregnant mouse and human myometrial contractility.	2011

Loss of functional K+ channels encoded by ether-à-go-go-related genes in mouse myometrium prior to labour onset.	2009
Dicer is required for female reproductive tract development and fertility in the mouse.	2009
A dominant loss-of-function GJA1 (Cx43) mutant impairs parturition in the mouse.	2009
The estrous cycle modulates small leucine-rich proteoglycans expression in mouse uterine tissues.	2009
Expression and function of K(v)7 channels in murine myometrium throughout oestrous cycle.	2009
Long form of leptin receptor gene and protein expression in the porcine trophoblast and uterine tissues during early pregnancy and the oestrous cycle.	2009
Expression of progesterone receptor membrane component 1 and its partner serpine 1 mRNA binding protein in uterine and placental tissues of the mouse and human.	2008
Estrogen and progesterone metabolism in the cervix during pregnancy and parturition.	2008
Delayed parturition and altered myometrial progesterone receptor isoform A expression in mice null for Krüppel-like factor 9.	2008

Overexpression of SK3 channels dampens uterine contractility to prevent preterm labor in mice.	2008
Activin-A in myometrium: characterization of the actions on myometrial cells.	2008
Infusion of oxytocin induces successful delivery in prostanoid FP-receptor-deficient mice.	2008
Side population in human uterine myometrium displays phenotypic and functional characteristics of myometrial stem cells.	2007
Are natural killer cells distributed in relationship to nerve fibers in the pregnant mouse uterus?	2007
Role of RYR3 splice variants in calcium signaling in mouse nonpregnant and pregnant myometrium.	2007
Expression of uterine sensitization-associated gene-1 (USAG-1) in the mouse uterus during the peri-implantation period.	2007
TRPC6.	2007
Overlapping and distinct expression of progesterone receptors A and B in mouse uterus and mammary gland during the estrous cycle.	2006
Signaling via the type I IL-1 and TNF receptors is necessary for bacterially induced preterm labor in a murine model.	2006

Ablation of connexin43 in uterine smooth muscle cells of the mouse causes delayed parturition.	2006
The estrogen-responsive adrenomedullin and receptor-modifying protein 3 gene identified by DNA microarray analysis are directly regulated by estrogen receptor.	2006
Change in prostaglandin E synthases (PGESs) in microsomal PGES-1 knockout mice in a preterm delivery model.	2005
Conditional deletion of beta-catenin in the mesenchyme of the developing mouse uterus results in a switch to adipogenesis in the myometrium.	2005
Interleukin-18 (IL-18) mRNA expression and localization of IL-18 mRNA-expressing cells in the mouse uterus.	2005
Dynamic cell type specificity of SRC-1 coactivator in modulating uterine progesterone receptor function in mice.	2005
Gestational-dependent changes in the expression of signal transduction and contractile filament-associated proteins in mouse myometrium.	2005
Oxytocin and estrogen receptor expression in the myometrium of pregnant relaxin-deficient (Rlx-/-) mice.	2005
Neonatal tamoxifen treatment of mice leads to adenomyosis but not uterine cancer.	2005

Carbonic anhydrase regulate endometrial gland development in the neonatal uterus.	2005
Identifying genetic networks underlying myometrial transition to labor.	2005
Uterine extracellular matrix components are altered during defective decidualization in interleukin-11 receptor alpha deficient mice.	2004
Differential activation of the connexin 43 promoter by dimers of activator protein-1 transcription factors in myometrial cells.	2005
Mammalian tachykinins and uterine smooth muscle: the challenge escalates.	2004
Gene expression profiling of neonatal mouse uterine development.	2004
Up-regulation of thymosin beta 4 gene expression in experimentally-induced uterine adenomyosis in mice.	2003
Expression, localization and function of prostaglandin receptors in myometrium.	2004
Differential expression of genes in the endometrium at implantation: upregulation of a novel member of the E2 class of ubiquitin-conjugating enzymes.	2003
Highly efficient and minimally invasive in-vivo gene transfer to the mouse uterus using haemagglutinating	2003

virus of Japan (HVJ) envelope vector.	
Inhibition of oxytocin receptor and estrogen receptor-alpha expression, but not relaxin receptors (LGR7), in the myometrium of late pregnant relaxin gene knockout mice.	2003
Diminished surface clustering and increased perinuclear accumulation of large conductance Ca2+-activated K+ channel in mouse myometrium with pregnancy.	2003
Bacterially-induced preterm labor and regulation of prostaglandin-metabolizing enzyme expression in mice: the role of toll-like receptor 4.	2003
Increase in the number of integrinbeta1-immunoreactive monocyte-lineage cells in experimentally-induced adenomyosis in mice.	2003
Microarray analysis of uterine gene expression in mouse and human pregnancy.	2003
Mouse Spam1 (PH-20) is a multifunctional protein: evidence for its expression in the female reproductive tract.	2003

Appendix 3

Mean Data ± SEM for Chapter 3

(A

	$MgSO_4$	Significance level
2mM	95.8 ± 1.1	ns
4mM	93.1 ± 1.6	ns
6mM	90.9 ± 2.3	*
8mM	86.2 ± 3.1	***
10mM	81.5 ± 3.5	***

(B)

	$MgSO_4$	Significance level
2mM	87.9 ± 4.9	ns
4mM	70.6 ± 5.0	***
6mM	62.7 ± 4.3	***
8mM	45.9 ± 5.7	***
10mM	31.2 ± 2.9	***

(C)

	$MgSO_4$	Significance level
2mM	94.6 ± 1.9	ns
4mM	105.2 ± 5.8	ns
6mM	104.4 ± 5.9	ns
8mM	104.6 ± 8.6	ns
10mM	108.9 ± 9.1	ns

(D)

	$MgSO_4$	Significance level
2mM	83.7 ± 4.4	ns
4mM	73.1 ± 4.2	***
6mM	57.7 ± 5.3	***
8mM	48 ± 5.1	***
10mM	40.1 ± 4.5	***

Changes in contractile properties of spontaneous contracting non-pregnant myometrium in response $MgSO_4$

Mean data showing concentration-dependent decrease in **(A)** amplitude force, **(B),** frequency, **(C)** duration and **(D)** AUC of spontaneously contracting non-pregnant myometrium. Values are expressed as Mean ± standard error mean (SEM). A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, **$p<0.01$, ***$p<0.0001$, ns is not significant

(A)

	$MgSO_4$	Significance level
2mM	92.1 ± 2.3	ns
4mM	81.8 ± 7.9	ns
6mM	67.8 ± 8.4	ns
8mM	49.8± 13	**
10mM	42.4 ± 12.5	***

(B)

	$MgSO_4$	Significance level
2mM	85.5 ± 2.2	ns
4mM	73 ± 3.5	**
6mM	52.7 ± 3.4	***
8mM	35.8 ± 7.4	***
10mM	23.7 ± 6.0	***

(C)

	$MgSO_4$	Significance level
2mM	84.7 ± 5.3	ns
4mM	77.8 ± 5.0	ns
6mM	71.6 ± 8.8	ns
8mM	59.2 ± 13.5	*
10mM	61.6 ± 13.6	*

(D)

	$MgSO_4$	Significance level
2mM	77.6 ± 4.7	**
4mM	61.9 ± 6.9	**
6mM	38.7 ± 6.0	**
8mM	25.0 ± 7.3	***
10mM	18.3 ± 6.3	***

Changes in contractile properties of spontaneous contracting day 14 pregnant myometrium in response $MgSO_4$

Mean data showing concentration-dependent decrease in **(A)** amplitude force, **(B),** frequency, **(C)** duration and **(D)** AUC of spontaneously contracting day 14 pregnant myometrium. Values are expressed as Mean ± SEM. A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, **$p<0.01$, ***$p<0.0001$, ns is not significant.

(A)

	$MgSO_4$	Significance level
2mM	96.2 ± 1.6	ns
4mM	94.3 ± 2.4	ns
6mM	92.9 ± 3.1	ns
8mM	75.1 ± 11.6	*
10mM	66.0 ± 15.0	**

(B)

	$MgSO_4$	Significance level
2mM	84.8 ± 12.3	ns
4mM	62.3 ± 9.1	ns
6mM	47.6 ± 5.2	*
8mM	31.0 ± 12.6	**
10mM	23.3 ± 11.7	**

(C)

	$MgSO_4$	Significance level
2mM	106.3 ± 7.4	ns
4mM	93.6 ± 5.6	ns
6mM	96.5 ± 12.6	ns
8mM	87.2 ± 13.5	ns
10mM	61.4 ± 23.1	ns

(D)

	$MgSO_4$	Significance level
2mM	86.0 ± 5.2	ns
4mM	64.3 ± 5.1	*
6mM	51.0 ± 4.6	**
8mM	27.5 ± 12.8	*
10mM	20.4 ± 11.6	*

Changes in contractile properties of spontaneous contracting day 16 pregnant myometrium in response to $MgSO_4$

Mean data showing concentration-dependent decrease in **(A)** amplitude force, **(B),** frequency, **(C)** duration and **(D)** AUC of spontaneously contracting day 16 pregnant myometrium. Values are expressed as Mean ± SEM. A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, **$p<0.01$, ***$p<0.0001$, ns is not significant.

(A)

	$MgSO_4$	Significance level
2mM	91.8 ± 2.7	ns
4mM	61.8 ± 10.9	*
6mM	56.9 ± 11.5	*
8mM	38.3 ± 9.9	***
10mM	19.6 ± 9.8	***

(B)

	$MgSO_4$	Significance level
2mM	72.5 ± 6.7	*
4mM	43.6 ± 8.2	***
6mM	35.5 ± 7.8	***
8mM	20.5 ± 7.1	***
10mM	9.6 ± 5.0	***

(C)

	$MgSO_4$	Significance level
2mM	110.0 ± 5.3	ns
4mM	76.8 ± 7.3	ns
6mM	74.6 ± 21.5	ns
8mM	64.4 ± 25.1	ns
10mM	44.3 ± 23.3	ns

(D)

	$MgSO_4$	Significance level
2mM	71.3 ± 6.4	**
4mM	43.3 ± 6.7	***
6mM	33.1 ± 6.5	***
8mM	18.0 ± 5.4	***
10mM	6.1 ± 3.2	***

Changes in contractile properties of spontaneous contracting term pregnant myometrium in response to $MgSO_4$

Mean data showing concentration-dependent decrease in **(A)** amplitude force, **(B),** frequency, **(C)** duration and **(D)** AUC of spontaneously contracting day 18 pregnant myometrium. Values are expressed as Mean ± standard error mean (SEM). A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, $**p<0.01$, $***p<0.0001$, ns is not significant

(A)

	$MgSO_4$	Significance level
2mM	98.7 ± 0.7	ns
4mM	96.7± 1.1	ns
6mM	95.0 ± 2.1	ns
8mM	92.7 ± 2.4	ns
10mM	90.8 ± 2.6	ns
12mM	89.3 ± 1.9	*

(B)

	$MgSO_4$	Significance level
2mM	85.3 ± 2.7	ns
4mM	66.1 ± 4.0	***
6mM	48.0 ± 5.0	***
8mM	40.0 ± 5.6	***
10mM	35.7 ± 5.5	***
12mM	36.13 ± 4.1	***

(C)

	$MgSO_4$	Significance level
2mM	103.8 ± 3.5	ns
4mM	93.6 ± 11.2	ns
6mM	92.6 ± 11.8	ns
8mM	77.7 ± 14.2	ns
10mM	82.1 ± 15.9	ns
12mM	92.8 ± 24.1	ns

(D)

	$MgSO_4$	Significance level
2mM	88.7 ± 2.6	**
4mM	73.4 ± 4.9	**
6mM	58.0 ± 6.1	**
8mM	54.2 ± 5.0	**
10mM	44.8 ± 5.21	***
12mM	38.87 ± 0.9	***

Changes in contractile properties of oxytocin-induced contracting non-pregnant myometrium in response to $MgSO_4$

Mean data showing concentration-dependent decrease in **(A)** amplitude force, **(B),** frequency, **(C)** duration and **(D)** AUC of oxytocin-induced contracting non-pregnant myometrium. Values are expressed as Mean ± standard error mean (SEM). A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, **$p<0.01$, ***$p<0.0001$, ns is not significant

(A)

	$MgSO_4$	Significance level
2mM	96.4 ± 1.3	ns
4mM	94.6 ± 1.5	ns
6mM	91.1 ± 2.4	ns
8mM	90.2 ± 2.2	ns
10mM	83.4± 4.1	ns
12mM	67.0 ± 11.4	***

(B)

	$MgSO_4$	Significance level
2mM	96.9 ± 1.5	ns
4mM	92.7 ± 2.3	ns
6mM	88.3 ± 4.8	ns
8mM	81.3 ± 4.1	ns
10mM	68.8 ± 7.2	**
12mM	51.3 ± 9.3	***

(C)

	$MgSO_4$	Significance level
2mM	101.5 ± 4.0	ns
4mM	109.5 ± 5.8	ns
6mM	97.2 ± 5.4	ns
8mM	96.1 ± 5.8	ns
10mM	99.6 ± 5.1	ns
12mM	74.0 ± 11.1	ns

(D)

	$MgSO_4$	Significance level
2mM	90.7 ± 2.4	**
4mM	86.3 ± 2.6	**
6mM	77.2 ± 3.5	***
8mM	68.9 ± 3.1	***
10mM	52.4 ± 5.4	***
12mM	34.9 ± 4.8	***

Changes in contractile properties of oxytocin-induced contracting day 14 pregnant myometrium in response to $MgSO_4$

Mean data showing concentration-dependent decrease in **(A)** amplitude force, **(B),** frequency, **(C)** duration and **(D)** AUC of oxytocin-induced of day 14 pregnant myometrium. Values are expressed as mean ± standard error mean (SEM). A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, **$p<0.01$, ***$p<0.0001$, ns is not significant.

(A)

	$MgSO_4$	Significance level
2mM	98.8 ± 1.0	ns
4mM	95.5 ± 1.2	ns
6mM	90.6 ± 1.7	ns
8mM	84.0 ± 2.0	*
10mM	71.3 ± 7.4	***
12mM	71.0 ± 4.9	***

(B)

	$MgSO_4$	Significance level
2mM	93.9 ± 2.3	ns
4mM	88.3 ± 3.2	ns
6mM	77.2 ± 3.2	***
8mM	65.7 ± 3.4	***
10mM	57.9 ± 3.3	***
12mM	58.0 ± 3.9	***

(C)

	$MgSO_4$	Significance level
2mM	101.4 ± 2.8	ns
4mM	109.7 ± 7.0	ns
6mM	106.8 ± 5.4	ns
8mM	109.3 ± 6.3	ns
10mM	100.9 ± 15.0	ns
12mM	112.2 ± 10.7	ns

(D)

	$MgSO_4$	Significance level
2mM	93.8 ± 1.2	ns
4mM	83.3 ± 1.5	**
6mM	73.6 ± 2.6	***
8mM	60.9 ± 3.5	***
10mM	50.5 ± 3.9	***
12mM	41.3 ± 6.7	**

Changes in contractile properties of oxytocin-induced contracting term pregnant myometrium in response to $MgSO_4$

Mean data showing concentration-dependent decrease in **(A)** amplitude force, **(B),** frequency, **(C)** duration and **(D)** AUC of oxytocin-induced contracting day 18 pregnant myometrium. Values are expressed as Mean ± standard error mean (SEM). A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, $**p<0.01$, $***p<0.0001$, ns is not significant